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# STUDIES ON STREPTOCOCCI

## V. A STUDY OF STREPTOCOCCAL PROTEINASES

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The known bacterial proteases produced by some but not all streptococci are gelatinase, discovered by Bitter,[1] fibrinolysin, discovered by Tillet and Garner,[2] and enzymes that hydrolyze casein and the M substance of group A streptococci recently purified and studied by Elliott.[3]

While a great deal of work has been done on these factors and their relation to antigenicity, little attention has been directed toward streptococcal enzymes capable of hydrolyzing serum and tissue globulins and albumins, and the potentialities these enzymes may have in relation to the disease-producing powers of a streptococcal strain.

The discovery by Bowman and Sherwood[4] that the factors described by Menkin[5] may be isolated from the tissues of rats, rabbits, and the embryonic chick ill with streptococcal infections focuses attention on streptococcal proteases. A toxic factor similar to necrosin has been isolated more recently by Watson and Cromartie from streptococcal lesions in rabbits.[6]

Apparently Eijkman[7,8] was one of the first to employ the auxanographic method of Beijerinck[9] in the study of proteolysis of casein by bacteria. This method consisted in the mixing of agar with some substrate to detect growth-promoting factors. Tongs[10] seems to be the first to study streptococcal proteolysis of casein by the plate method. He reported that 7 of 19 hemolytic streptococci hydrolyzed casein, while 4 out of 26 green streptococci developed clearing around the colonies on milk agar plates.

Stevens and West,[11] using the test

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2. Tillet, W. S. and Garner, R. L. 1933, The fibrinolytic activity of hemolytic streptococci. J. Exper. Med. **58**: 485–502.
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5. Menkin, V. 1948, Newer Concepts of Inflammation, Springfield, Illinois, Charles C Thomas.
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10. Tongs, M. S. 1919, Ectoenzymes of streptococci. J. A. M. A. **73**: 1277–1279.
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tube method, reported that extracts of certain streptococci ground with glass were able to hydrolyze peptone and casein but had no effect upon denatured horse albumin. They also investigated the kinetics of the reactions.

Frobisher[12] was the first to demonstrate a proteinase which he called "histase," which attacked cooked beef and human tissues, as well as casein. Todd[13] repeated Frobisher's experiments and confirmed his findings. Elliott,[14] using the test tube method and a group A streptococcus, demonstrated streptococcal proteinase for casein, fibrin, gelatin, and the M substance. In 1950 Elliott[3] reported upon the crystallization and serological differentiation of a streptococcal proteinase for casein and its precursor. This work was followed by electrophoretic studies of the proteinase and its precursor by Shredlovsky and Elliott[15] in 1951.

In previous studies Sherwood, Wahl, Colglazier, Hamilton, and Jay[16] and Sherwood, Russell, Bowman, and Ott[17] have discussed the possibility that enzymes or antienzymes produced either by the streptococcus or the host might play a role in streptococcal infection. They report that they could see no correlation between the production of hyaluronidase and the $LD_{50}$ for the mouse and the chick. Elliott[14] suggested that the absence of the proteinase for casein was correlated with virulence for mice. This work has led the authors to make an extensive study of proteolytic enzymes that might attack casein, partially denatured egg albumin, human albumin, and human $\gamma$ globulin. So far as we know no studies of the proteolysis of egg albumin, human albumin, and human $\gamma$ globulin by streptococcal proteinases have been made other than a preliminary report on two of these by Luzzio and Sherwood.[18]

The present paper is an attempt to answer by experiment the following questions:

1. If an agar plate containing the various substances mentioned is inoculated on the surface with representatives of the various Lancefield groups, will the colonies produce zones of clearance either aerobically or anaerobically? Furthermore, would such zones of clearence be truly indicative of proteolysis?

2. What is the effect of thioglycollate in the medium upon the clearing phenomenon under both aerobic and anaerobic conditions? What is the effect of sodium sulfide, glutathion, and cystein on either growth or the clearing reaction on human $\gamma$ globulin proteose peptone agar?

3. Do the data suggest that more than one proteinase is produced by streptococci?

12. Frobisher, M., Jr. 1926, Tissue-digesting enzyme (histase) of streptococci. J. Exper. Med. **44**: 777–785.
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17. Sherwood, N. P., Russell, B., Bowman, K. and Ott, J. 1952, Studies on streptococci. IV. A study of the relationship of certain virulence factors in streptococcal infections to the $LD_{50}$ dose of streptococci. J. Infect. Dis. **91**: 246–259.
18. Sherwood, N. P. and Luzzio, A. J. 1951, Streptococcal albuminase. Navy Task Order #7 Annual Report, pp. 20–27.

4. Is there any correlation between the presence or absence of the enzyme for any substrate and the $LD_{50}$ for the mouse or the embryonic chick?

5. Can one employ the data obtained to divide the streptococci belonging to any one of the Lancefield groups into subgroups?

## MATERIALS AND METHODS

*Basal medium.*—The basal medium employed was proteose-peptone agar (Difco), modified to the extent that PP#2, 0.2% dextrose, and 2% agar were employed.

*Thioglycollate agar.*—To the basal medium was added sodium thioglycollate to give a final concentration of $10^{-3}M$.

*Substrates employed were as follows:*

1. Grade A raw skimmed milk.

2. Crystalline egg albumin prepared by the method of Kekwick and Cannon.[19]

3. Pure human albumin and $\gamma$ globulin were obtained from the Massachusetts Department of Health, The University Laboratory of Physical Chemistry related to Medicine and Public Health, Harvard University, and from Squibb and Sons through the courtesy of the Red Cross. The $\gamma$ globulin contained pyrogens which rendered it unsuitable for clinical purposes.

*Human albumin agar.*—A 7.5% purified human albumin solution was prepared in distilled water and adjusted to pH 5.8. The solution was sterilized by Seitz filtration. The sterile solution was then denatured by immersion in an 80 C water bath for 4 minutes. The denatured albumin was aseptically transferred to a sterile Waring blendor and homogenized for 5 minutes. The homogenate, largely foam, was aseptically transferred to a sterile 250 ml Erlenmeyer flask and stored at 6 C overnight. This allowed the foam to break. Ten ml of the homogenate were transferred to 90 ml of sterile liquid basal-medium agar, thoroughly mixed, and poured into Felsen quadrant-dishes.

*Casein agar.*—Skim milk was autoclaved at 10 lb for 15 min. Ten ml of the skim milk were transferred to a bottle of agar and poured as described for human albumin-agar.

*Egg albumin agar.*—A 5% solution of crystalline egg albumin was prepared with distilled water and suspended in agar as described for human albumin-agar.

*Human $\gamma$ globulin agar.*—A 5% solution of human $\gamma$ globulin was prepared with physiological saline and $\gamma$ globulin agar plates prepared as described for human albumin.

*Thioglycollate substrate agar.*—Thioglycollate agar was prepared containing sodium thioglycollate in a final concentration of $10^{-3}$ *M*.

*Quadrant plates.*—The protein-agar mediums were poured into Felsen dishes so that the agar was level with the quartering sections of the Felsen dishes, or 5 mm in depth. The agar was allowed to solidify, the plates inverted, and placed in the incubator at 37 C for 24 hours to dry and to test for sterility.

*Inoculum.*—The drop from a 1 ml tuberculin syringe with a 26 gauge, $\frac{3}{4}$ inch needle was used as a standard inoculum. A drop of organisms from a 24-hour culture in proteose peptone #2 broth was placed in the center of each quadrant, so as to produce an inoculum zone 8 mm in diameter.

*Incubation.*—The plates were incubated at 37 C for 96 hours.

*Anaerobiosis.*—For anaerobic incubation the plates were placed in Brewer's anaerobic jars and the tops sealed with plasticene. The air was evacuated from the jar and replaced with nitrogen. This procedure was repeated three times to insure that the oxygen had been flushed out.

*Grouping.*—The cell suspensions which were to be used to inoculate the plate were first grouped, and then the plates were inoculated. After 96 hours incubation, colonies were picked from the plates, subcultured, and finally regrouped. The grouping was performed according to instructions received from the Communicable Disease Center, Chamblee, Georgia,[20] except that Difco proteose peptone #2 was used instead of the Difco tryptose phosphate broth suggested.

*pH of the hydrolyzed zone.*—The clear zones were excised with a number 10 cork borer, macerated in a minimum quantity of distilled water (about 10 ml), and the pH reading taken with a Beckman line operated pH meter. The same amount of uncleared agar from outside the clear zone was treated in the same manner.

*Determination of proteolysis.*—The Sorensen[21] formol titration, the Cu-titration, the biuret method, and the Herriott[22] modification of the

19. Kekwick and Cannon, described by Hawk, P. B., Oser, B. L. and Summerson, W. H. 1947, Practical Physiological Chemistry, ed. 12, Philadelphia, Blakiston Co., p. 172.

20. Personal communication.

21. Sorenson, S. P. L., see Hawk, Oser, and Sommerson, 1949, Practical Physiological Chemistry, ed. 12, Philadelphia, Blakiston Co., 879.

22. Herriott, R. M., see Northrop, Kunitz, and

Folin-Ciocalteu method were investigated for possibilities as an adequate technique for the estimation of proteolysis. It was experimentally shown that the Herriott-Folin-Ciocalteu procedure was most sensitive for our experimental conditions. The technique was that described by Northrop, Kunitz and Herriott[22] and also by Hawk, Oser and Sommerson.[19] Briefly, an equal volume of 10% trichloracetic acid is added to an aliquot of sample contained in a calibrated centrifuge tube. After mixing, the sample is allowed to stand for 15 minutes and is then centrifuged. To an aliquot of the supernatant is added an equal volume of the Folin-Ciocalteu chromogenic reagent. The color is allowed to develop for 5 minutes, and the intensity is measured in optical density units in a Coleman Universal spectrophotometer, at 660 mμ. The color intensity is directly proportional to the degree of protein hydrolysis.

*Streptococci employed.*—Strain 1195 was obtained from Elliott and strain 1068 from Dr. R. G. E. Murray. Two strains were isolated from an epidemic in guinea pigs and one from an equine infection. The remaining organisms were lyophilized cultures of strains isolated from clinical human cases. The source of most of them has been reported by Sherwood, Russell, Bowman, and Ott.[17]

## RESULTS

In order to obtain data to be used in developing answers to the first three questions outlined above, batches of milk, purified egg albumin, human albumin, and human γ globulin agars, with and without thioglycollate, were prepared and poured into quadrant plates. Duplicate plates for both aerobic and anaerobic cultivations were inoculated with each of the 50 hemolytic streptococci being studied and incubated for 96 hours at 37 C, when the results to be found in table 1 were recorded. In the case of γ globulin, additional plates were prepared to determine the effect of sodium sulfide, glutathione and cysteine on growth and the clearing phenomenon. All of the experiments have been repeated several times, care being taken to check for purity and Lancefield grouping of the suspension being inoculated and finally of the resulting growth on plates.

An inspection of table 1 not only brings out clear-cut answers to the first three questions being investigated, but raises other questions. When casein is used as a substrate, all of the representatives of groups A, B, C, and D grew. Of the 21 group A streptococci cultured aerobically on casein agar plates, 11 produced definite zones of clearing (fig. 1 and 2), 1 produced a small zone, and 9 produced no zone of clearing. Under anaerobic conditions 12 produced as large or larger zones of clearing than when grown aerobically, while 5 produced smaller zones, and only 4 failed to produce any evidence of hydrolysis. Anaerobiosis seemed essential for hydrolysis by 4 strains.

The results obtained when streptococci belonging to groups B and C were inoculated onto casein agar plates are in marked contrast with those of the above or, for that matter, with 14 group D streptococci. None of the group B or C streptococci produced more than a very small zone of clearing. However, 2 of the 6 resembled several group A in that they did not produce any clearing on casein or any other substrate employed. Thioglycollate seemed to have no effect on the casein proteinases of groups B and C. One notices a striking difference in results when group D streptococci are grown on casein agar plates. All 14 streptococci produced large zones of clearing (Fig. 2). When thioglycollate was added to the medium the only noticeable effect was a tendency to re-

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Herriott, 1948, Crystalline Enzymes, ed. 2, New York, Columbia University Press, p. 309.

See also Reaction of Folin's reagent with proteins and biuret compounds in presence of cupric ions. Proc. Soc. Exp. Biol. Med., 1941, **46**: 642–644.

TABLE 1.—*Results of aerobic and anerobic cultivation of streptococci on proteose peptone #2 agar with various protein substrates and with or without thioglycollate.*

| Group | Strain no. | Casein | | | | Egg albumin | | | | Human albumin (SII) | | | | Human γ globulin (SIII) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Aerobic | | Anaerobic | | Aerobic | | Anaerobic | | Aerobic | | Anaerobic | | Aerobic | | Anaerobic | |
| | | Plain | Thio. | Plain | Thio. | Plain | Thio. | Plain | Thio. | Plain | Thio. | Plain | Thio. | Plain | Thio. | Plain | Thio. |
| A | 1062 | ++ | +++ | +++ | +++ | s.g. | − | ± | ± | + | − | ± | ± | − | − | + | − |
| | 1074 | ++ | +++ | +++ | +++ | s.g. | − | ± | ± | + | − | + | ± | s.g. | − | ± | − |
| | 1004 | ++ | − | +++ | +++ | − | − | ± | ± | + | − | − | − | − | − | ± | − |
| | 1042 | ++ | − | +++ | +++ | − | − | ± | ± | − | − | + | − | − | − | + | − |
| | 1069 | ++ | ++ | +++ | +++ | s.g. | s.g. | + | + | + | − | + | ± | − | − | + | ± |
| | 1003 | ± | + | +++ | +++ | − | − | ± | ± | − | − | − | − | − | − | + | − |
| | 1075 | ++ | − | ++ | + | s.g. | s.g. | ± | ± | − | − | + | ± | − | − | + | ± |
| | 1100 | ++ | ++ | +++ | ++ | − | − | + | ± | + | − | + | ± | − | − | + | − |
| | 1063 | ++ | ++ | +++ | ++++ | s.g. | s.g. | ± | ± | + | − | + | − | − | − | − | − |
| | 1070 | ++ | ++ | ++ | ++ | − | − | + | + | − | − | − | − | − | − | ± | − |
| | 1068 | ++ | − | ++ | +++ | − | − | + | + | + | − | + | ± | + | − | − | − |
| | 1195 | + | − | ++ | ++ | 0 | 0 | − | − | ± | − | − | − | − | − | ± | ± |
| | 1067 | − | − | ± | ± | s.g. | s.g. | − | − | − | − | − | − | − | s.g. | s.g. | − |
| | 1072 | − | − | − | − | − | − | − | − | − | − | − | − | − | s.g. | − | − |
| | 1049 | − | − | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1046 | − | − | ± | − | − | − | − | − | − | − | − | − | − | − | s.g. | − |
| | 1061 | − | ± | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1064 | − | − | − | − | − | − | − | − | − | − | − | − | − | s.g. | − | − |
| | 1054 | − | − | − | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1044 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1071 | − | − | + | ± | | | | | − | − | − | − | − | − | − | − |
| B | 1076 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1078 | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1079 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1210 | ± | ± | + | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1002 | ± | − | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1048 | ± | − | ± | − | − | − | − | − | − | − | − | − | − | − | − | − |
| C | 1083 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1084 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1085 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1086 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1059 | ± | ± | + | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1081 | ± | ± | + | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1056 | ± | ± | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1058 | ± | ± | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| | 1082 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| D | 1021 | ++++ | ++++ | +++ | ++ | − | − | − | − | +++ | ++ | ++ | ++ | ++++ | +++ | +++ | +++ |
| | 1026 | +++ | ++++ | +++ | ++ | − | − | − | − | ++ | + | ++ | + | +++ | +++ | +++ | +++ |
| | 1192 | +++ | +++ | +++ | ++ | − | − | ± | ± | +++ | + | ++ | ++ | ++++ | ++++ | +++ | +++ |
| | 1105 | +++ | +++ | +++ | ++ | − | − | − | − | ++ | + | + | + | +++ | ++ | +++ | +++ |
| | 1093 | +++ | +++ | ++ | ++ | − | − | ± | ± | +++ | + | + | + | +++ | ++++ | +++ | +++ |
| | 1080 | ++++ | +++ | +++ | +++ | ± | ± | ± | ± | +++ | + | + | + | ++++ | ++++ | ++++ | +++ |
| | 1092 | +++ | ++ | +++ | ++ | ± | ± | ± | ± | ++ | + | + | + | +++ | ++++ | ++++ | +++ |
| | 1041 | ++++ | ++++ | +++ | ++ | ± | ± | ± | ± | ++ | + | ++ | + | ++++ | +++ | +++ | +++ |
| | 1090 | +++ | +++ | +++ | ++ | − | − | − | − | +++ | + | +++ | + | ++++ | ++++ | +++ | +++ |
| | 1091 | +++ | +++ | +++ | ++ | ± | ± | ± | ± | +++ | + | +++ | + | ++++ | +++ | ++++ | +++ |
| | 1008 | ++++ | ++++ | ++ | + | ± | ± | ± | ± | ++ | + | +++ | ++ | ++++ | +++ | ++++ | +++ |
| | 1039 | ++++ | ++++ | ++ | +++ | ± | ± | ± | ± | ++ | + | +++ | ++ | +++ | +++ | ++++ | +++ |
| | 1017 | +++ | +++ | ++++ | ++ | ± | ± | ± | ± | +++ | + | ++ | ++ | ++++ | +++ | +++ | +++ |
| | 1405 | +++ | +++ | ++ | +++ | ± | ± | ± | ± | +++ | + | ++ | + | ++++ | ++++ | +++ | +++ |

0, no growth.
s.g., scanty growth.
−, growth but no clearing.
±, growth and clearing of 0.5 to 1.0 mm on each side of growth area.
+, growth and clearing of 1.5 to 4.0 mm on each side of growth area.
++, growth and clearing of 4.5 to 7.0 mm on each side of growth area.
+++, growth and clearing of 7.5 to 10.0 mm on each side of growth area.
++++, growth and clearing of 10.0 to 15.0 mm on each side of growth area.

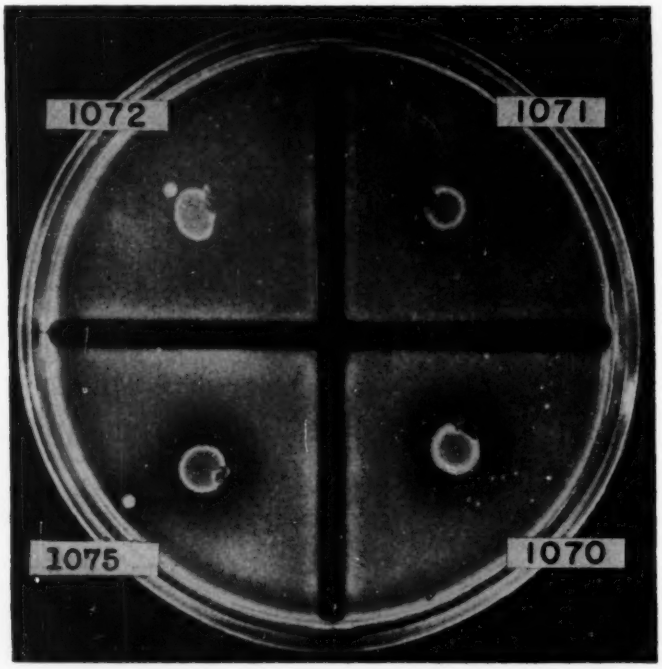


FIG. 1.—Proteolysis of casein. Group A, streptococci 1070, 1071, 1072, and 1075.

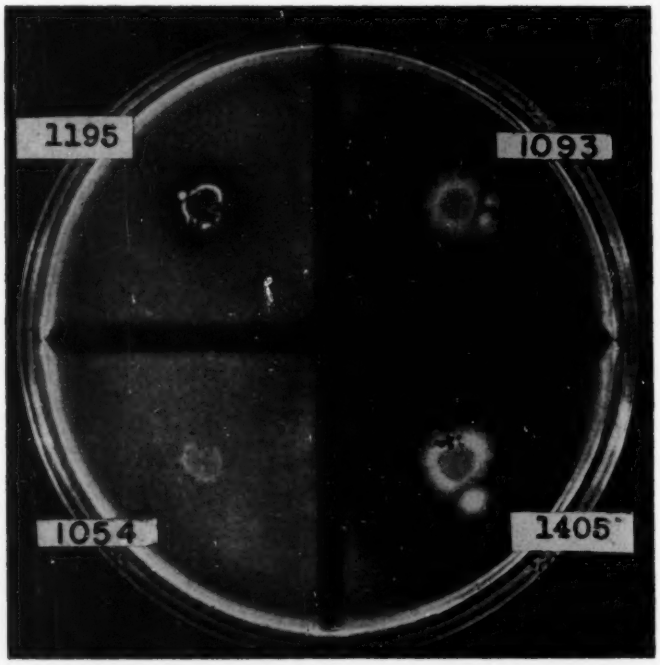


FIG. 2.—Proteolysis of casein. Group A, streptococci 1195 and 1054. Group D, streptococci 1093 and 1405.

duce or inhibit the amount of clearing on the plates grown under anaerobic conditions.

When partially denatured egg albumin was used as a substrate, Elliott's strain no. 1195 did not grow aerobically but grew anaerobically with the production of a moderate zone of definite clearing. Six strains produced only scanty growth aerobically, which was affected only slightly, if at all, by thioglycollate. Anaerobically, 4 group A strains produced definite zones of clearing, and 8 produced very small zones on plain egg albumin plates both with and without thioglycollate. The remaining strains produced growth but no zones of clearing. Thus it will be seen that anaerobiosis definitely enhanced growth of 7 and stimulated hydrolysis of partially denatured egg albumin by 12 strains of group A, while thioglycollate had little, if any, demonstrable effect. None of Lancefield's group B and C streptococci produced clearing on egg albumin plates, but all grew well.

Eight of the 14 group D streptococci produced small zones of clearing with or without thioglycollate when cultured aerobically on egg albumin plates. Eleven produced similar results when grown under anaerobic conditions.

When partially denatured human albumin was used as a substrate, all strains of groups A, B, C and D grew well under both aerobic and anaerobic conditions. Under aerobic conditions, 1 of the 21 group A strains produced a very small zone of clearing or partial clearing, while 7 strains produced zones designated as one plus. The remaining 13 strains did not develop zones of clearing on human albumin agar plates incubated under aerobic conditions. The presence of thioglycollate in the medium completely inhibited hydrolysis. When similar plates were incubated under anaerobic conditions, the results indicate that anaerobiosis did not increase hydrolysis and that thioglycollate tends to inhibit the latter.

When groups B and C streptococci are grown aerobically or anaerobically on partially denatured human albumin agar plates either with or without thioglycollate, the results are uniformly positive for growth but without zones of clearing.

Fourteen strains of group D inoculated onto human albumin agar plates not only grew well, but produced definite zones of hydrolysis under both aerobic and anaerobic conditions. When thioglycollate is present in the medium, hydrolysis shows marked retardation under both aerobic and anaerobic conditions.

When $\gamma$ globulin is used as a substrate, all strains grew. Of the 21 group A strains, only 1 possessed the ability to produce, as a rule but not always, a small amount of hydrolysis under aerobic conditions, and 10 when grown anaerobically. The general effect of thioglycollate was to reduce hydrolysis. None of the strains of group B and C produced hydrolysis of human $\gamma$ globulin, although all grew well. In marked contrast to these results are those obtained with 14 group D streptococci. In the experiment reported in table 1, all produced 3 and 4+ hydrolysis that was not noticeably affected by the presence of thioglycollate in the $\gamma$ globulin. In repeat experiments the degree of hydrolysis was frequently 2 or 3+.

In addition to experiments where thioglycollate was added to $\gamma$ globulin, other experiments were completed in which sodium sulfide, glutathione, and cystein, respectively, were substituted for thioglycollate. These substances all contain sulfhydryl groups, and the results obtained were similar to those in which thioglycollate were employed.

As mentioned earlier in the paper, an

TABLE 2.—*Effect of pH on streptococcal hydrolysis of human serum albumin.*

| | *a* Uninoculated control | | | *b* Group D streptococci +#1093 | | |
|---|---|---|---|---|---|---|
| Incubation hours → | 0 (albumin + buffer) | 96 (albumin + buffer) | | 0 (Strep. + albumin + buffer) | 96 (Strep. + albumin + buffer) | |
| Initial pH | Optical density* | | Final pH | Optical density* | | Final pH |
| 5.0 | 0.02 | 0.03 | 5.7 | 0.07 | 0.12 | 5.8 |
| 6.0 | 0.02 | 0.02 | 6.0 | 0.09 | 0.13 | 6.0 |
| 7.0 | 0.02 | 0.02 | 6.8 | 0.095 | 0.335 | 6.5 |
| 8.0 | 0.02 | 0.028 | 7.8 | 0.10 | 0.38 | 7.6 |
| 8.5 | 0.025 | 0.033 | 8.5 | 0.12 | 0.44 | 7.8 |
| 9.0 | 0.025 | 0.04 | 8.8 | 0.13 | 0.43 | 7.9 |
| 9.5 | 0.03 | 0.025 | 9.2 | 0.14 | 0.47 | 7.2 |
| 10.0 | 0.03 | 0.035 | 9.4 | 0.135 | 0.42 | 7.3 |

Both *a* (uninoculated control) and *b* (streptococci #1093) contained: 2 ml 2% albumin and 8 ml buffer at the various pH's. In addition, 2 ml of physiological saline were added to *a* and 2 ml of 12.5% (w/v) cells were added to *b*.
Buffer—sodium diethylbarbituate.
Temperature—37 C.
* Color developed by Folin-Ciocalteu reagent (Herriott) and read at 660 mμ. The optical density is directly proportional to the degree of proteolysis.

TABLE 3.—*Streptococcal hydrolysis of serum albumin.*

| | Uninoculated control | Streptococci #1093 | |
|---|---|---|---|
| Flask no. | 1 (Albumin + saline) | 2 (Strep. + albumin) | 3 (Strep. + saline) |
| Time in hours | Optical density* | | |
| 0 | 0.009 | 0.06 | 0.058 |
| 24 | 0.03 | 0.17 | 0.098 |
| 48 | 0.012 | 0.235 | 0.065 |
| 72 | 0.027 | 0.29 | 0.085 |
| 96 | 0.022 | 0.315 | 0.07 |
| 120 | 0.085 | 0.35 | 0.075 |
| 144 | 0.035 | 0.40 | 0.077 |

Flasks 2 and 3 contained 10 ml of 2.5% bacterial cells. Flasks 1 and 2 contained 2 ml of 2% albumin. Final volumes made to 12 ml with saline where needed.
pH—7.4 (phosphate buffer, 0.055 *M*).
Temperature—37 C.
* Color developed by Folin-Ciocalteu reagent (Herriott) and read at 660 mμ. Optical density is directly proportional to degree of proteolysis.

important point to be resolved was whether the zone of clearing around areas of growth of some of the streptococci represented hydrolysis, or was it due to nonenzymatic peptization or dispersion of the colloidal particles.

In an attempt to answer this, it was decided to determine whether pH changes in the medium could be a factor in clearing. Accordingly, a series of 8 flasks of broth suspensions of streptococci containing pH values ranging from 5 to 10 was incubated at 37 C. Controlled flasks containing albumin and physiological saline and buffer and adjusted to corresponding pH were incubated under the same conditions. Samples were removed at 0 and 96 hours from each of the flasks, and evidence of hydrolysis was determined by Herriott's[22] colorimetric procedure, using a Coleman Universal spectrophotometer, at 660 mμ. The results are given in table 2. The color intensity is directly proportional to the degree of hydrolysis. The control flasks showed no change between pH 5 and pH 7, and only moderate change of a gradual increase in hydrolysis from pH 7 to pH 10. The flask containing bacteria, however, showed definite and increasing amounts of hydrolysis at 96 hours, beginning with pH 5 and being quite marked at pH 7 and 8. This would indicate then that the medium would have to have a pH of between 7.5 and 8 to show even slight evidence of hydrolysis after incubation for 96 hours. Further evidence that clearing is due to hydrolysis is to be found in table 3.

Following this, plates of human albumin agar were prepared with the various streptococci and incubated for 96 hours. After this, the clear zones were excised with a no. 10 cork borer, and the material was macerated in about 10 ml of distilled water having a pH of 7, and pH readings were taken. The same amount of uncleared agar from outside the cleared zone was macerated and suspended in 10 ml of distilled water, and the pH was determined. Typical results are recorded in table 4.

An inspection of table 4 shows that the pH in the area of hydrolysis was not responsible for the clearing. Our results indicate that clearing around the areas of growth on protein agar plates is due to hydrolysis of the partially denatured protein.

The report by Elliott[14] that the failure

TABLE 4.—*Comparison of proteolysis (plate method) of purified human albumin under aerobic conditions by various Lancefield groups and pH values of zone of clearance.*

| Strain | Group | Hydrolysis | pH of hydrolized zone | pH of unhydrolized zone |
|---|---|---|---|---|
| 1062 | A | + | 7.2 | 7.35 |
| 1074 | A | + | 7.2 | 7.3 |
| 1195 | A | ± | 7.3 | 7.35 |
| 1072 | A | − | | 7.4 |
| 1049 | A | − | | 7.4 |
| 1064 | A | − | | 7.4 |
| 1021 | D | + + + | 6.5 | 7.1 |
| 1026 | D | + + + | 6.5 | 7.1 |
| 1192 | D | + + + + | 6.6 | 7.1 |
| 1105 | D | + + + + | 6.47 | 7.1 |
| 1093 | D | + + + + | 6.4 | 7.1 |
| 1041 | D | + + + | 6.4 | 7.1 |

−, growth but no clearing.
+, growth and clearing of 0.5 to 4.0 mm on each side of growth area.
+ +, growth and clearing of 4.5 to 7.0 mm on each side of growth area.
+ + +, growth and clearing of 7.5 to 10.0 mm on each side of growth area.
+ + + +, growth and clearing of 10.5 or more mm on each side of growth area.

of group A streptococci to produce proteolysis of casein correlated with virulence led us to compare the virulence of 7 group A, 2 group C and 4 group D streptococci for the mouse and the embryonic chick with ability to hydrolyze casein and human albumin, respectively. The results are given in table 5. A careful inspection of the table reveals little, if any, correlation between avirulence for either the mouse or embryonic chick and either proteolysis or the lack of proteolysis of casein, human albumin, or human γ globulin. In this connection it is interesting to note that in a previous communication by Sherwood, Russell, Bowman and Ott[17] it was stated that group A strain 1061, which produces a little hydrolysis of casein under anerobic conditions, was obtained from a case of erysipelas, and strains 1059 and 1081 of group C, which also produce a caseinase, were the cause of a fatal epidemic in guinea pigs.

## DISCUSSION

Bacterial proteases and peptidases are as yet poorly characterized and understood enzymes. Much of the interpretation of the data in this study must be made on the basis of knowledge of proteolytic enzymes from other sources, namely higher plant and animal enzymes.

It is interesting to note that proteolytic activities vary among the Lancefield-grouped streptococci. Generally, the group characteristics vary from the highly proteolytic group D to the relatively inert groups B and C; group A organisms are somewhat intermediate between the actively proteolytic D cells and the inert B and C streptococci.

The streptococci described in this

TABLE 5.—*Comparison of hydrolysis of casein and human albumin with the $LD_{50}$ for the mouse and the embryonic chick.*

| Group | Strain | Aerobic hydrolysis | | | Anaerobic hydrolysis | | | Mouse virulence | Chick virulence |
|---|---|---|---|---|---|---|---|---|---|
| | | Casein | Albumin | γ globulin | Casein | Albumin | γ globulin | | |
| A | 1046 | − | − | − | ± | − | − | Avirulent | |
| | 1068 | + + | + | − | + + | + | − | $10^{0}$ | $10^{-0.4}$ |
| | 1070 | + + | − | − | + + | − | ± | $10^{-0.4}$ | $10^{-2}$ |
| | 1071 | − | − | − | + | − | − | $10^{0}$ | |
| | 1072 | − | − | − | − | − | − | $10^{-1}$ | $10^{-3}$ |
| | 1074 | + + | + | − | + + + | + | ± | $10^{0}$ | |
| | 1061 | − | − | − | ± | − | − | $10^{-1}$ | $10^{-2}$ |
| C | 1059 | ± | − | − | + | − | − | $10^{-3.4}$ | $10^{-3}$ |
| | 1081 | ± | − | − | + | − | − | $10^{-3.0}$ | $10^{-3}$ |
| D | 1008 | + + + + | + + | + + + + | + + | + + + | + + + + | $10^{0}$ | $10^{-1}$ |
| | 1017 | + + + | + + + | + + + + | + + + + | + + | + + + | $10^{0}$ | $10^{-2}$ |
| | 1090 | + + + | + + + | + + + + | + + + | + + + | + + + + | Avirulent | $10^{-2}$ |
| | 1190 | | + + + + | | | + + + | | $10^{-2.5}$ | $10^{-2}$ |

−, growth but no clearing.
+, growth and clearing of 0.5 to 4.0 mm on each side of growth area.
+ +, growth and clearing of 4.5 to 7.0 mm on each side of growth area.
+ + +, growth and clearing of 7.5 to 10.0 mm on each side of growth area.
+ + + +, growth and clearing of 10.5 or more mm on each side of growth area.

paper have at least two, and perhaps three or more, distinct proteolytic enzymes. For want at present of better terminology, these enzymes may be referred to as "caseinases," "albuminases" and "globulinases."

The proteolytic activity of group A streptococci against casein which is demonstrated by the plate method is confirmed by colorimetric analysis. Not all members of group A are active against casein, but where proteolytic activity is observed, there is a tendency toward greater activity under anaerobic conditions. The somewhat greater anaerobic caseinase activity of the group A cells is suggestive of an enzyme sensitive to oxidizing agents. These results relative to a group A streptococcal caseinase are in agreement with those reported by Elliott and Dole[23] and Elliott.[3] Insofar as egg albumin is concerned, there is but slight activity against the protein; this activity is confined to but a few strains. This activity, where observed, is found under anaerobic conditions both in the presence and absence of thioglycollate. Group A cells have a small degree of proteolytic activity against human albumin, and there is virtually no activity against human $\gamma$ globulin. Such activity as is found occurs anaerobically; thioglycollate either has no effect or is somewhat inhibitory for proteolysis. For group A streptococci, then, under the experimental conditions described, there is good evidence for the existence of only a caseinase. There is some suggestion in the data that it may be of the papain group, insofar as necessity for reducing groups is concerned. There is just enough of a hint of proteolysis of egg albumin, human albumin, and human $\gamma$ globulin to warrant extension of investigations into such factors as pH optimums, metal or other cofactor activations.

The latter statement is likewise applicable to the action against casein by groups B and C. While but indifferent activity is observable by some members of these groups, there is some suggestion in the data that caseinases may be present.

In contrast to groups A, B, and C, group D streptococci present an actively proteolytic pattern. Group D cells actively hydrolyze casein under both aerobic and anaerobic conditions. There is virtually no effect of thioglycollate aerobically. There is some diminution by thioglycollate of anaerobic proteolysis as compared to the aerobic activity. Whether these differences are real is being determined during the course of investigations on the quantitative nature of proteolysis by the streptococci. In similar fashion, the possibility of metallo-components, or components sensitive to either oxidation or reduction is being studied. Comparison of group A caseinase with group D caseinase tends to indicate that we may be dealing with two different types of caseinases, for group D caseinase activity is virtually unchanged by aerobic or anaerobic conditions, in contrast to group A caseinase. Whether A caseinase and D caseinase cleave the same peptide bond positions within the protein is at present under investigation, employing synthetic peptide substrates.

While group D has little or no activity on egg albumin, human albumin is hydrolyzed aerobically as well as anaerobically. Under aerobic conditions, thioglycollate diminishes proteolysis; anaerobic proteolysis is quantitatively less than aerobic hydrolytic activity, and thioglycollate has either no or slight effect on anaerobic proteolysis. Enzymatic degradation of albumin is apparently more sensitive to thioglycollate

23. Elliott, S. D. and Dole, V. P. 1947, An inactive precursor of streptococcal proteinase. J. Exper. Med. **85**: 305–320.

than is the corresponding breakdown of casein. This is suggestive of a component, possibly metal in nature. A seeming difference between the proteolysis of the two substrates, human albumin and casein, also would seem to indicate that in addition to a caseinase there is also present in group D streptococci an albuminase. The hint of albuminolytic activity by some members of group A cells certainly raises the question: are we dealing with a proteolytic enzyme system which is complete in group D but incomplete in group A, but is otherwise identical?

Human $\gamma$ globulin is vigorously digested by group D cells, in contrast to the other three groups studied. There are occasional quantitative differences observed between anaerobic and aerobic proteolysis. These differences can be more definitely described following experiments of a quantitative nature. Thioglycollate is either neutral or slightly inhibitory in its effect. The data seem to point to the presence of an additional enzyme, a globulinase, in the group D streptococci.

In all of the experiments reported, some role of reducing groups or metallo groups seems to be indicated. Sulfhydryl containing enzymes are, of course, inactivated by oxidation, and this type seems to be present in group A organisms. Metallo-groups are in similar fashion inactivated by -SH groups; evidence of this type of enzyme is likewise found in the present studies. Many proteolytic enzymes are unaffected by either metals or reducing or oxidizing groups, and this type also seems to be present in some of the cells studied.

Future reports will deal with more purified streptococcal proteinases, so that any activating and inactivating factors may be better defined. A highly purified casein will be employed in these studies. The nature of the protein cleavage will also be described.

The results reported in this paper and those by Ott and Sherwood[24] on pharmacological studies of whole cultures and streptococcal supernatants are being extended, with an idea of throwing light upon the phenomena observed. While streptococcal proteinases and other products of streptococci investigated apparently do not correlate with virulence, they may play a role in streptococcal disease. Since Sherwood et al[17] found that none of the so-called virulence factors are individually or collectively the lethal ones in streptococcal infections, it is possible that the work in progress expanding the pharmacological studies mentioned above may throw light upon the latter. It is conceivable that necrosin and other toxic factors isolated from the tissues of infected animals may result from the liberation or activation of the hosts own enzymes by some product of the bacteria produced in vivo.

## SUMMARY AND CONCLUSIONS

It has been established that the clearing phenomenon around colonies of streptococci on casein, albumin, and gamma globulin agar plates is indicative of hydrolysis of the substrate. The pH of a zone of clearing is, as a rule, lower than that of the surrounding medium. The optimum initial pH for hydrolysis of human albumin of group A streptococci is between 7.0 and 7.5. The optimum initial pH for group D albuminase seems to be close to 9.5, although the reaction quickly drops to between 6.8 and 7.4. The beginning of nonenzymatic peptization due to the pH is close to 8.0.

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24. Ott, J. L. and Sherwood, N. P. 1950, Pharmacological reactions produced in rabbits and mice by streptococcal products. Proc. Soc. Am. Bact., p. 86.

A survey of table 1 indicates that groups A, B, and C streptococci can be subdivided into those that hydrolyze casein and those that cannot under the conditions of the experiment. All of 14 group D streptococci hydrolyzed casein, human albumin, and $\gamma$ globulin. Seven of the fourteen group D streptococci produced a slight amount of hydrolysis of egg albumin. Table 1 also shows that all the streptococci grew on casein, egg albumin, human albumin, and $\gamma$ globulin mediums. In striking contrast to the moderate hydrolytic activity of many group A and the strong hydrolytic activity of all group D streptococci, Lancefield's groups B and C were quite inert, showing only slight activity toward casein and were inactive when grown on the other substrates studied.

The results suggest that several proteinases may be produced by streptococci. It is suggested that these be designated as caseinases, albuminases, and globulinases. Work is in progress to determine whether such assumptions are valid.

No correlation was found between virulence for the mouse or the chick and the ability to hydrolyze any of the substrates. Such results are not interpreted as indicating that streptococcal proteinases play no role in streptococcal disease.

# STUDIES ON THE PASSAGE OF HELMINTH LARVAE THROUGH HOST TISSUES

## I. HISTOCHEMICAL STUDIES ON THE EXTRACELLULAR CHANGES CAUSED BY PENETRATING LARVAE

## II. ENZYMATIC ACTIVITY OF LARVAE IN VITRO AND IN VIVO

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Penetration of the skin or mucosa followed by migration through connective tissue into the host vascular system is a necessary part of the life cycle of many species of parasitic helminths. During their brief and often rapid passage through the skin, the infective larvae must traverse the nonliving stratum corneum, the cellular layers of the epidermis and the extracellular barriers presented by the basement membrane and ground substance of the dermal connective tissue. The basement membrane is found just below the stratum germinativum as a continuous, optically homogeneous nonfibrillar sheet in which reticular fibers are embedded. It is considered to be continuous with the acellular ground substance of the dermis.[1] The basement membrane and ground substance also act as barriers to any larvae whose routes of infection entail passage through other epithelia or through connective tissue. Many infective larvae have no obvious mechanical means of effecting entrance but they do possess glandular structures which have been cited by various investigators as probable sources of secretions which aid in penetration. In addition, detailed cytological studies of the changes associated with penetration, particularly with cercariae of *Schistosoma mansoni* strongly support this hypothesis.[2–5] However, except for a preliminary report by the present authors,[6] we know of no other histochemical studies on the

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6. Lewert, R. M. and Lee, C. L. 1951, The effect of helminths on the basement membrane and ground substance of the host: a study of the mechanism of penetration. J. Parasitol. **37** (sec. 2): 20.

effect of penetrating parasites on the acellular elements of the connective tissue. Most of the work to be presented in this report concerns the effect of a variety of helminths on their hosts' skin, particularly those changes induced in the extracellular or intercellular substances during the first few minutes after the parasite contacts the skin surface. Having assumed that there would be a softening of these acellular materials with formation of soluble products if parasite enzymes act on these substances, we have utilized histochemical methods designed for the preservation and visualization of these products. These methods are particularly valuable for the study of changes in the basement membrane and ground substance (*Section I, A*).

There is evidence that the acellular elements of the connective tissue exist in states of polymerization that vary with age, growth, and other conditions which affect host physiology.[1,7–9] These substances are thought to depolymerize or become more soluble under certain of these conditions and may also be similarly changed when acted upon by suitable enzymes such as bacterial collagenase. It seems probable that with changing conditions of the host, the acellular substances might vary in their resistance to penetration by helminth larvae. Accordingly, various factors affecting the state of these acellular elements of the host have been studied to ascertain whether these changes are reflected in the ability of the parasites to penetrate (*Section I, B*).

Various tests have been utilized by investigators in an attempt to determine the type of enzymatic acivity possessed by penetrating helminths. In some cercariae (*Diplostoma* and *Cryptocotyle*), relatively crude tests suggest the presence of proteolytic activity[10,11] Preparations of schistosome-infected snail tissue have been shown to exert lytic effects on skin sections.[4] A "spreading factor" has been described in adult nematodes (*Ancylostoma*), schistosome cercariae and stylet cercariae (*Cercaria ocellata* and *C. pseudarmata*).[12–14] Hyaluronidase-like activity has been described in extracts of cercariae of *S. mansoni*[15–17] and in stylet cercariae.[14] Other investigators have been unable to demonstrate this enzyme in cercariae of *S. mansoni*.[4] It has also been suggested that the lipolytic activity of larvae of *Nippo-*

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16. Stirewalt, M. A. and Evans, A. S. 1952, Demonstration of an enzymatic factor in cercariae *of S. mansoni* by the streptococcal decapsulation test. J. Infect. Dis. **91**: 191–197.
17. Evans, A. S. 1953, Quantitative demonstration of hyaluronidase activity in cercariae of *Schistosoma mansoni* by the streptococcal decapsulation test. Exper. Parasitol. **2**: 417–427.

*strongylus muris* is related to their ability to penetrate the skin.[18] In the present investigation, a comparative study of enzymatic activity has been made using several methods in an attempt to relate such activity to the effects of larvae on the acellular elements of connective tissue (*Section II*).

## SECTION I. HISTOCHEMICAL STUDIES ON THE EXTRACELLULAR CHANGES CAUSED BY PENETRATING LARVAE

Of the three histochemical methods most used in this investigation, two have been utilized extensively in critical studies of the basement membrane and ground substance of connective tissue.[1,7] These are the Hotchkiss (periodic acid-leucofuchsin) technique and the technique of using Evans blue intravenously as a vital stain. The third technique, which we have referred to as the ferrocyanide technique, demonstrates the amount of free or solvent water in any extracellular site. It was developed by Dr. S. Permutt* and has not previously been described. All three techniques demand the use of freezing-drying methods of fixation, embedding and section preparation in order to satisfactorily preserve the acellular elements with which we are concerned. Many of the alterations in these elements are due to the production of soluble or mobile substances which are lost or which shift in position with the usual techniques of fixation and section preparation.

The Hotchkiss technique, used with proper controls, identifies polysaccharide-containing protein.[19] It has been suggested that the intensity of the staining reaction is related to the state of organization of this glycoprotein,† possibly to its degree of polymerization or aggregation. Changes in this state, in the nature of a depolymerization, can be brought about by mucase enzymes. Such changes may result in a loss of material and a decrease in staining intensity. Other enzymes such as bacterial collagenase can also bring about a less intense staining reaction. In some instances, an increase in the intensity of staining is found in highly polymerized material after enzymes act on the ground substance. As this process continues, however, the material becomes more diffusely or less intensely stained. This is probably due to the removal or loss of the soluble products thus formed. The loss may continue until affinity for the stain is also lost.

The Evans blue technique complements the above method. It has "an almost diagnostic affinity for the areas of formation of water soluble, alcohol-insoluble carbohydrate-containing protein."[7] Normally, little Evans blue is bound by the ground substance. In general, where an enzyme is acting on a Hotchkiss-positive substrate to form more reactive groups, we can expect to find deeper staining with Evans blue. The ferrocyanide technique gives additional information. When the ground substance is altered and becomes less resistant to passage of larvae, one would expect that more water would be present in the extracellular phase. The ferrocyanide technique is a method for demonstrating the presence and relative amount of free extracellular water in connective tissue.

18. Thorsen, R. E. 1953, Studies on the mechanism of immunity in the rat to the nematode *Nippostrongylus muris*. Am. J. Hyg. **58:** 1–15.

* Research Associate, Department of Anatomy, University of Chicago.

19. Hotchkiss, R. D. 1948, A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations Arch. Biochem. **16:** 131–141.

† For convenience, the terms polysaccharide-containing protein and glycoprotein are used synonymously throughout this paper to refer to the Hotchkiss-positive substances not removed by treatment with saliva or methyl alcohol chloroform (see methods).

## SECTION I. A. STUDIES OF PENETRATION IN NORMAL ANIMALS

### *Materials and general methods*

The penetration of the cercariae of *S. mansoni*, *Schistosomatium douthitti*, and of the filariform larvae of *Ancylostoma caninum*, *Strongyloides ratti*, *Strongyloides simiae* and *N. muris* has been studied in detail. The penetration of the infective larvae of *Trichinella spiralis* as they pass through the mucosa of the duodenum and of the next generation as they enter muscle was also intensively studied. The passage of various other stages through tissues was also investigated, e.g., the penetration of the snail by miracidia and the movement of schistosome eggs through the intestinal epithelium.

The skin penetration studies were made on white rats (Sprague-Dawley) and mice (CF1) and a comparison was made of young (3 month) and old (12 to 18 month) animals. In general, older animals (6 to 8 month) were used in preference to younger ones as their basement membranes are more sharply defined and stain relatively intensely with the Hotchkiss technique. In any experiments where comparisons between animals were made, care was taken to use animals of the same age, sex, strain and approximate weight. The dorsal skin was used in preference to that of the abdomen and if a single area was to be exposed, the interscapular skin was usually chosen. This area has been shown to have a relatively even basement membrane of consistent thickness.[1] The hair was removed with electric clippers, shaved with the aid of mild soap and later carefully washed to remove any traces of soap. If any evidence of new or old trauma was found, the area was not used. Depilatories were not used since they could not be sufficiently controlled and were often found to cause more trauma to the skin than careful shaving.

The methods of isolation and concentration of larvae will be discussed in *Section II*. The number of larvae used varied from experiment to experiment. However, for most of the routine studies 200 to 500 larvae of *Schistosoma*, 500 to 1000 larvae of *Ancylostoma*, or 2000 to 5000 larvae of *Strongyloides* or *Nippostrongylus* were used for each area of the skin being tested. Whenever comparisons are made, the larvae in question have been handled in the same way, i.e., they are from the same culture pool, have been isolated at the same time and subsequently treated in the same way, and the same approximate number of larvae has been used in the exposure of each area, etc., in order to eliminate differences due to variations in viability or infectivity of the larvae being used. Cercariae of *S. mansoni* were confined to specific areas by the use of rubber wells (finger cots with the finger end clipped off, held tight to the shaved skin by perforated adhesive tape). Other larvae were allowed to penetrate without restriction.

The principle method used in preparing tissues was the freezing-drying technique.[20] In this procedure, tissues are removed as rapidly as possible after the death of the animal and plunged into isopentane at −150 C. They are then dehydrated in vacuum while being maintained at −30 C to −40 C (the major portion of this work was done at −30 C). Subsequent to this dehydration the tissues are embedded in paraffin under vacuum. In some cases, duplicate material was embedded directly in nitrocellulose after freeze-drying for cytological study. Control preparations were also made by the usual methods of fixation in Zenker-formol followed by nitrocellulose embedding, and were stained with hematoxylin eosin azure II for comparison with the histochemical preparations. Serial sections were made with either rotary or sliding microtomes.

*The Evans blue technique.*—The basic method used was the administration of Evans blue intravenously at the rate of 1 ml of a 1% solution of the dye in 0.85% saline per 100 g body weight. This was very satisfactory for making photographic records since intense color developed. The penetration sites described in the results can readily be demonstrated by using concentrations of even less than one-tenth of this standard. In every case the dye was given via the caudal vein while the animals were under nembutal anesthesia. Infective larvae of the various parasites were placed on the skin or given via stomach tube, depending on the species, at regular intervals, e.g., 15, 30, and 60 minutes prior to decapitation. Fifteen or 20 minutes after the dye was injected the animals were killed by decapitation. As soon after death as possible, the tissues were removed and frozen in isopentane. Sections were made of tissues at 15 $\mu$, mounted with gentle finger pressure on lightly albuminized or unalbuminized slides, passed through two changes of Schillaber's low viscosity immersion oil in Coplin jars at 60 C, and covered with a coverslip for examination. The warm immersion oil removes the paraffin and clears the specimen but does not cause any shift in the site of the Evans blue.

*The Hotchkiss technique.*—The slides were prepared from frozen-dried tissues embedded in

20. Gersh, I. 1948, Application in pathology of the method of fixation by freezing and drying of tissues. Internat. A. M. Museums Bull. **28:** 179–185.

paraffin as described. The paraffin was removed from the sections by immersion in three changes of anhydrous petroleum ether. The sections were transferred to absolute ethyl alcohol for a suitable period (usually 16 hours) prior to staining. The original method of Hotchkiss was used, as well as several variations on the standard technique, such as regulation of the pH of the periodic acid solution and the leucofuchsin at 3.5 and substitution of alcoholic solutions in other stages of the technique.[21] Sections were prepared 10 $\mu$ or 15 $\mu$ in thickness and control sections were treated with phosphate buffer and buffered saliva at pH 7.0. Other control sections were treated with methyl alcohol chloroform at 60 C for 6 hours. In this way the relative amount of water-soluble material, glycogen and phospholipid in the connective tissue could be deduced.

*The ferrocyanide technique.*—The ferrocyanide technique as developed by Permutt was used. A 20% solution of $Na_4Fe(CN)_6 \cdot 10H_2O$ was injected intravenously at the rate of 0.5 ml per 100 g body weight 5 minutes before the animal was killed by a blow on the head. The tissues were prepared by freezing-drying as described above. The paraffin was removed from these sections by placing the slides in a 1% solution of anhydrous ferric chloride (2 changes) in xylene, then in two changes of 1% anhydrous ferric chloride in absolute ethyl alcohol, followed by rapid passage through water containing 1% ferric chloride. The sections were then dehydrated by rapid passage through absolute alcohol to xylene and mounted in resin. In this way, as the paraffin was removed, the highly soluble sodium ferrocyanide was precipitated by reaction with the ferric chloride to form Prussian blue (ferric ferrocyanide).

Some frozen-dried tissue sections were also examined for metachromasia. After removal of paraffin by petroleum ether and air drying, these slides were gently flooded with 0.01% toluidine blue, covered with a slip and examined immediately.

## *Results*

*Schistosomes.*—The results derived from experiments with cercariae of both *S. mansoni* and *S. douthitti* are essentially identical as far as the histochemical study is concerned. Because of this, no distinction is made between them in this portion of the text.

21. Permutt, S. and Johnson, F. B. 1953, Histochemical studies on the lens following radiation injury. Arch. Path. **55**: 20–30.

Evans blue. Gross findings.—Immediately after the intravenous injection of Evans blue in the quantities routinely utilized in this study, the skin appears faintly tinged with blue due to circulating dye. Within 2 or 3 minutes after cercariae are placed on the shaved skin, blue spots appear. These minute spots become larger and more prominent within the next few minutes, while those which first appeared become dark blue with a lighter blue halo. By the end of 15 minutes the site of penetration usually has attained its maximum color and is demonstrable as a blue area from one to several millimeters in diameter. This state is illustrated in plate I, figures 1 and 2. If many larvae are placed on the same area the blue spots may become confluent (plate I, fig. 1 and 3). In some individuals, maximum color is not developed until approximately 30 minutes after the cercariae are placed on the skin. It is interesting to note that these organisms (as well as others to be mentioned later) tend to concentrate or are able to penetrate first at the periphery of the exposed area, i.e., at the junction of the skin and the suspending water. This was even more commonly seen when rubber dams (finger cots) were used to retain the water containing the larvae.

If cercariae are allowed to penetrate the skin at increasing intervals of time, e.g., 15, 30, 60 and 90 minutes prior to the injection of Evans blue, a most surprising condition becomes apparent. Only the areas exposed for 15 and 30 minutes show the blue spots at the penetration site, and although 1 or 2 spots may be visible on the 60 minute area, none is ever seen in the area on which cercariae have been placed 90 minutes before dye injection. This pattern is illustrated by plate I, figures 1 to 4. This sequence may be demonstrated by exposing different areas on the same

animal or by exposing comparable areas on different animals of the same strain and age. If Evans blue is injected 16 to 24 hours after penetration of the skin, the area penetrated, which is slightly edematous, appears to retain a little more dye than the surrounding area but can barely be distinguished from the normal.

Evans blue. Microscopic.—Sections of skin removed during the first few minutes of penetration show the Evans blue to be localized about the penetrating cercariae. In some instances the ground substance of the dermis immediately beneath the larva binds blue dye even before the cercaria has left the cellular layers of the epidermis. At 15 minutes the ground substance surrounding the larvae in the dermis is deeply stained, and by 30 minutes the entire dermal ground substance in the general area being penetrated is pale blue, with deeply staining areas around the immediate sites of the penetrating larvae. In tissues fixed 30 to 45 minutes after larvae had been placed on the skin surface, no extensive bluing of the upper dermis remains but occasionally the lower dermis is diffusely stained, and when larvae can still be found no intense binding of the dye occurs in their immediate vicinity. Sections made of skin fixed 60 minutes after larvae were placed on the surface differ in no way from the control areas with respect to Evans blue retention. Following the penetration of large numbers of cercariae, one occasionally sees congested dermal capillaries. However, usually no such change is seen and little or no dye is retained in the lumen of the superficial dermal capillaries. The sequence of events just described is illustrated semidiagrammatically in plate II. Following penetration of the skin by parasites we do not ususally find specific structures retaining the dye. However, in several instances where cercariae have been seen just as they are passing through the basement membrane, the membrane at this point appears to be stained blue. Also, elastic fibers of the dermis sometimes appear to retain the dye. The stained material resulting from cercarial penetration is readily removed from the section if it is passed through an alcohol series to water. Occasionally, a very small amount remains immediately adjacent to the penetrating organism. This possibly represents material just being altered by the parasite's secretions but not as yet soluble.

Hotchkiss technique.—In sections of

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PLATE I

FIG. 1–5—Evans blue: Gross appearance of the dorsal skin of normal adult white rats following intravenous dye injection.

FIG. 1.—Result of exposure to *S. ratti* and *N. muris*. Evans blue staining material is present in the areas penetrated by *Strongyloides* at 15 and 30 minutes exposure to larvae and is no longer perceptible at 60 and 90 minutes. (This is essentially identical to the results seen following exposure to schistosome cercariae.) The areas exposed to *N. muris* give no response. ×1.

FIG. 2–4—Results of exposure of three rats to *S. ratti* for 15, 30, and 90 minutes. In the 15 minute exposure (fig. 2) individual penetration sites may still be seen; at 30 minutes the entire exposed area (fig. 3) is blue; at 90 minutes (fig. 4) no blue is retained but a wheal persists. The border of the wheal is indicated at two points by the arrows. ×4.

FIG. 5.—The effect of double exposure of the same area of skin. Area A exposed to *S. ratti* 60 minutes before injection of dye; Area B exposed 60 and 15 minutes before injection of dye. ×4.

FIG. 6.—Thin film of gelatin on glass stained with hematoxylin after exposure to helminth extracts and controls (0.01 ml for 2 hours at 20 C). Only the upper row is considered positive. The area exposed to water alone has swollen and retained more dye. ×1.

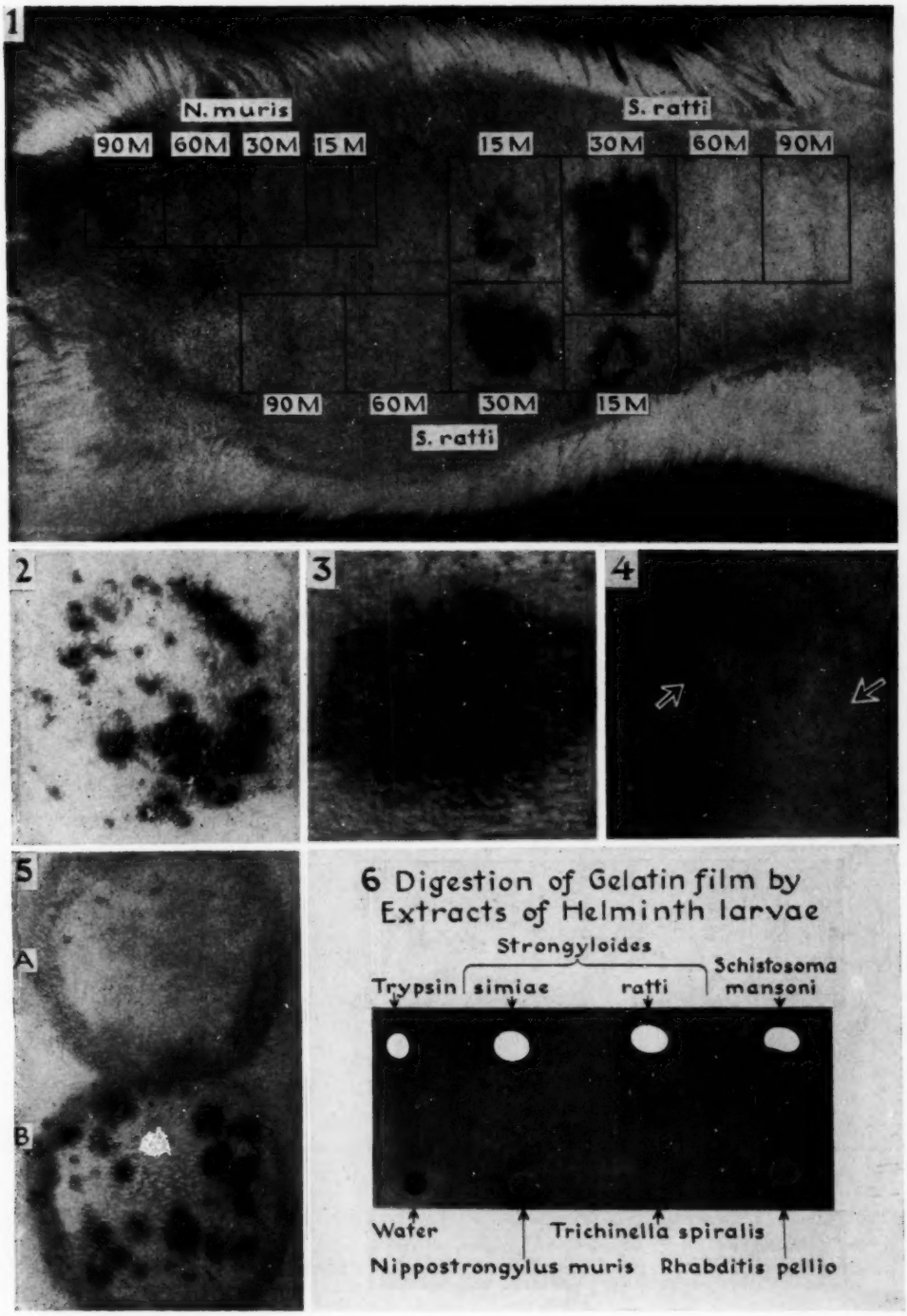

1
N. muris
90M 60M 30M 15M
S. ratti
15M 30M 60M 90M
90M 60M 30M 15M
S. ratti
2
3
4
5
A
B
6 Digestion of Gelatin film by Extracts of Helminth larvae
Strongyloides
Trypsin
simiae
ratti
Schistosoma mansoni
Wafer
Nippostrongylus muris
Trichinella spiralis
Rhabditis pellio

PLATE I

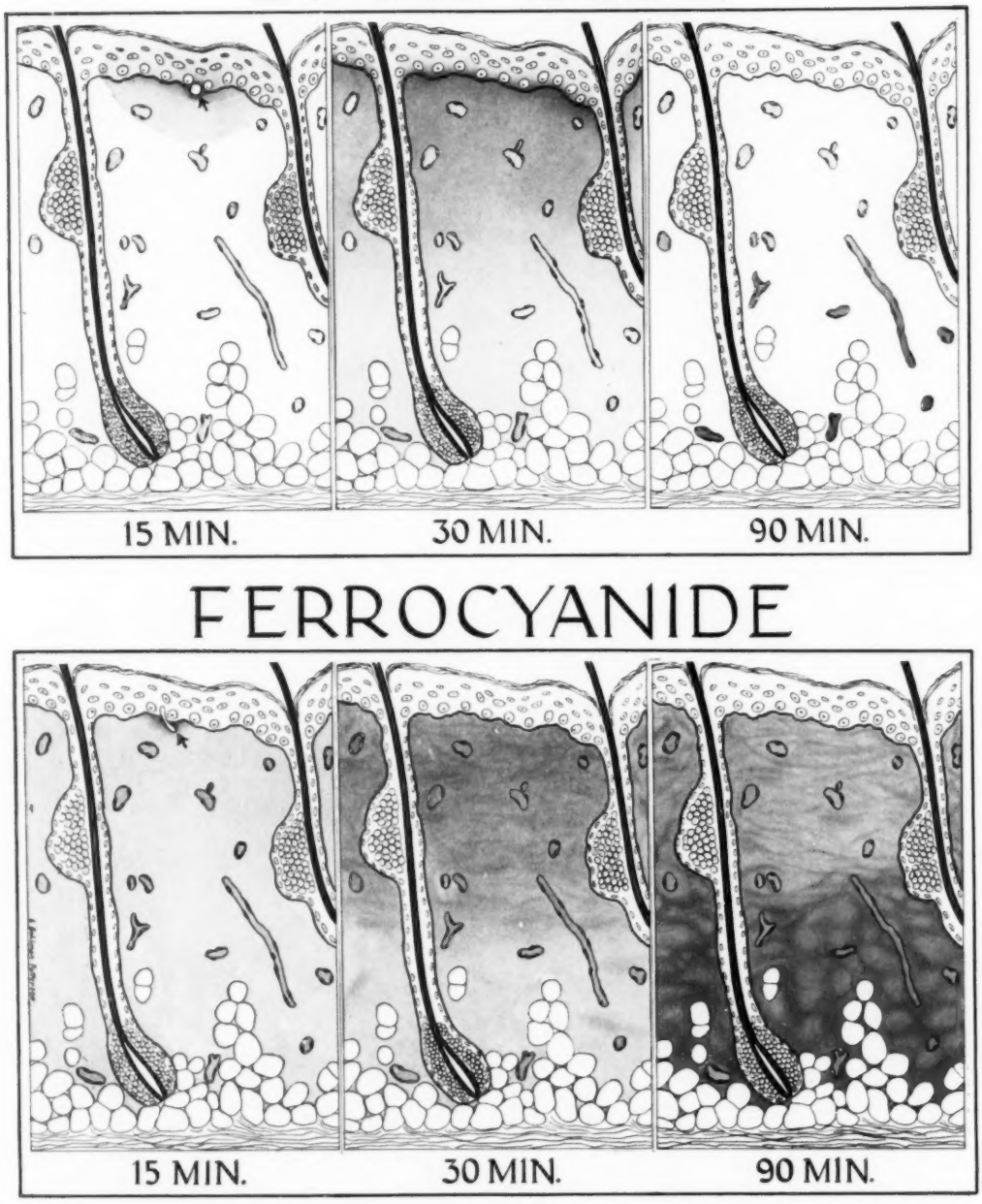



PLATE II

Diagrammatic representation of the alterations of ground substance during the first 90 minutes of skin penetration as shown by the Evans blue and ferrocyanide techniques.

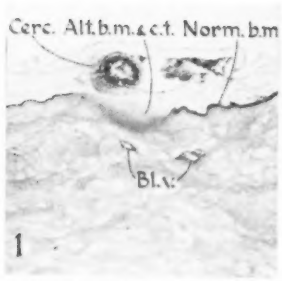
Evans blue series.—Increase and subsequent disappearance of Evans blue-staining material in the ground substance (soluble glycoprotein).

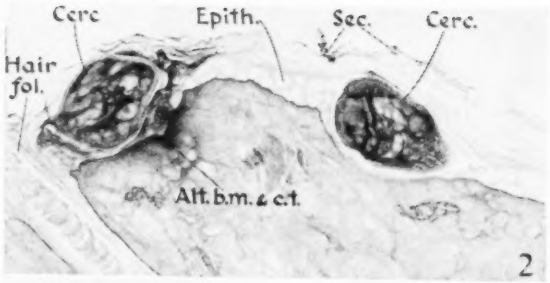
Ferrocyanide series.—Increase in free water of the ground substance with increase in time of action of larvae.

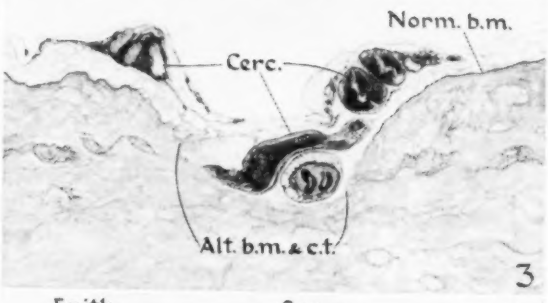
15, 30, 90 min.—length of time elapsed since larvae were placed on skin surface.

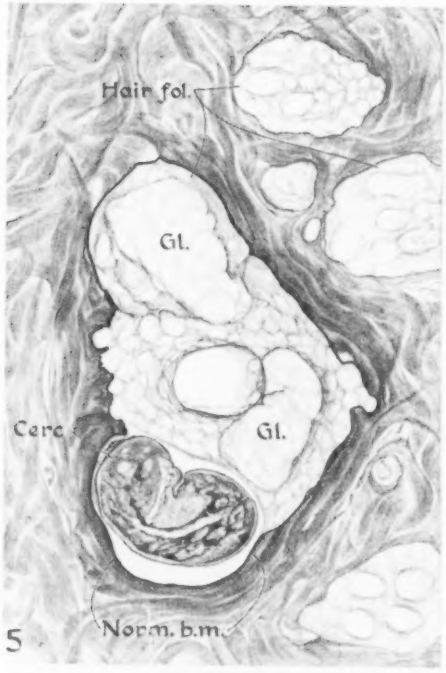
Arrow—indicates approximate position of larva at 15 minutes.

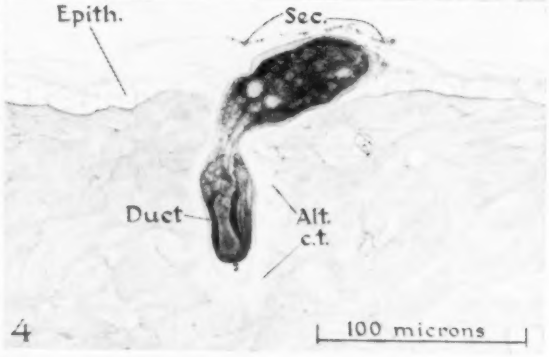
No attempt has been made to include in the diagram those alterations which simultaneously occur in the basement membrane.

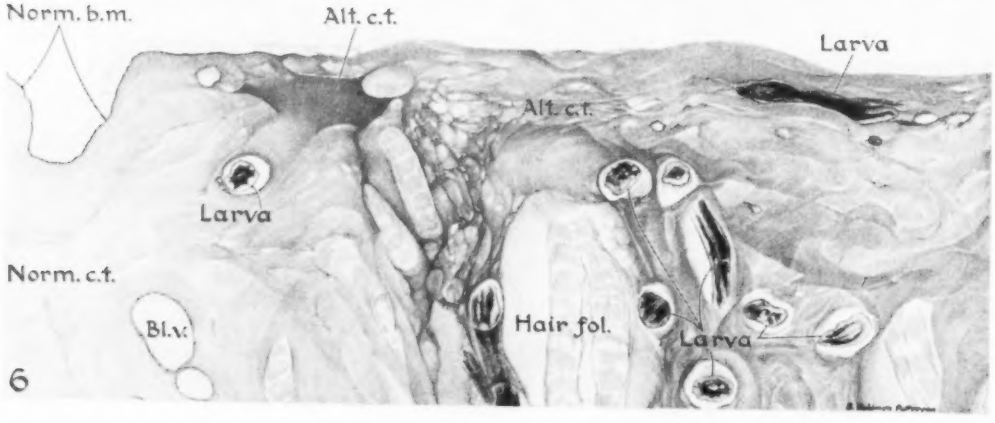

Cerc.
Alt.b.m.& c.t.
Norm.b.m
Bl.v.
1


Cerc
Epith.
Sec.
Cerc.
Hair fol.
Alt. b.m. & c.t.
2


Norm. b.m.
Cerc.
Alt. b.m. & c.t.
3


Hair fol.
Gl.
Gl.
Cerc
Norm. b.m.
5


Epith.
Sec.
Duct
Alt. c.t.
4
100 microns


Norm. b.m.
Alt. c.t.
Larva
Alt. c.t.
Larva
Norm. c.t.
Bl.v.
Hair fol.
Larva
6

tissue prepared by this method, the cercariae are readily seen due to the presence of relatively large amounts of Hotchkiss-positive material. Some of this is glycogen or other material soluble in buffer or buffered saliva. However, the most prominent features of both species are the intensely stained ducts of the lateral penetration glands. These may be seen in plate III, figure 4. The cercariae apparently discharge part of the contents of these glands on the skin surface. This polysaccharide-containing material appears granular while within the organism and is often seen as stringy or filamentous masses on the skin surface. The secretion on the skin is not preserved by usual methods but can be readily seen in frozen-dried preparations (see plate III, figs. 1 to 4). The cercariae penetrate the stratum corneum and make tunnels parallel to

PLATE III

Alteration of the basement membrane and ground substance of rat skin during penetration by helminth larvae as shown by the Hotchkiss technique.

FIG. 1.—Fifteen-minute penetration by *S. mansoni* in 6-month-old rat showing disappearance of basement membrane prior to penetration of the dermis.

FIG. 2.—Fifteen-minute penetration by *S. douthitti* in 6-month-old rat with color increase in ground substance (at left) and disappearance of basement membrane (cercaria at right).

FIG. 3.—Fifteen-minute penetration by *S. mansoni* with disappearance of basement membrane as cercaria (center) enters the dermis.

FIG. 4.—Thirty-minute penetration by *S. douthitti* in 6-month-old rat showing color decrease around anterior end of cercaria.

FIG. 5.—*S. mansoni* cercaria retained in hair follicle 24 hours after exposure of 12-month-old rat. Note lack of alteration in basement membrane.

FIG. 6.—Fifteen-minute penetration by *A. caninum* in 6-month-old rat. Note general color increase in ground substance and disappearance of basement membrane except for normal area at left of figure. (Scale on this figure applies to entire plate.)

Cerc.—cercaria
Alt. bm. and C. T.—altered basement membrane and ground substance of the dermal connective tissue.
Norm. B. M.—normal basement membrane.
Norm. c.t.—normal connective tissue.
Bl.v.—dermal capillary.
Hair fol.—hair follicle.
Epith.—stratum germinativum.
Sec.—Hotchkiss-positive cercarial secretion.
Duct.—duct of cercarial penetration gland.
Gl.—sebaceous glands of hair follicle.
All material fixed by freezing-drying method, sectioned at 15 $\mu$.
The figures were drawn with the aid of a camera lucida by E. Bohlman Patterson.

the skin surface. During this period, more of this Hotchkiss-positive secretion may be seen in the tunnels, often around or just below the organism (see plate III, fig. 1). These tunnels through the stratum corneum are easily seen in rat skin but they are not so readily observed in the mouse, where the corneum is relatively thin. We commonly find cercarial tails drawn well into the tunnels in this layer of the skin. Within as little as 3 minutes after cercariae are placed on the skin surface, they may come to rest immediately above the basement membrane. The basement membrane on which the parasite may sometimes be seen to rest is often extensively altered. It is obvious that the glycoprotein material is affected by this penetration. Within the first few minutes after the cercaria comes in contact with it, the membrane may appear to be somewhat thickened and more diffusely stained. The general picture is one of increased coloration, usually limited to an area 1 or 2 times the diameter of the cercaria. This stage, illustrated in plate III, figures 1 and 2, is apparently transient. More often we find that the basement membrane has completely disappeared or is unstained in the immediate vicinity of the organism, as illustrated in plate III, figures 2 to 4. This unstained area varies in extent from a relatively small locus approximately the diameter of the cercaria to an area roughly 2 or 3 times the cercarial diameter. Similar changes may also be seen in the ground substance although they are not as prominent. The normal ground substance stains a relatively even, diffuse pink but that in the vicinity of a cercaria is more lightly colored so that in favorable specimens the anterior end of the cercaria in the dermis appears to be surrounded by a lighter halo (plate III, fig. 4).

The Hotchkiss-positive material present in the glands and in the ducts of the cercariae can best be seen in cercariae that have not as yet emerged from the snail. In these it is occasionally possible to see 6 pairs of cells filled with granular polysaccharide-containing material and 6 ducts filled with this substance (plate IV, fig. 3 and 6). Four can usually be readily demonstrated. After emergence from the snail, only 3 ducts can be seen and some of the glands have been exhausted of their content (plate IV, fig. 4). Much of the remaining material in the glands is applied to the surface of the skin or is discharged while the cercariae pass through the epidermis (plate III). By the time the cercariae reach the dermis the glands of most individuals are free of polysaccharide although some remains within the ducts (plate III, fig. 4, plate IV, fig. 5). We have not observed any appreciable amount of this substance in the dermis in the vicinity of the cercariae although it is conceivable that some small portion may be released after penetration of the basement membrane.

Study of hematoxylin-stained slides shows that as a result of the passage of the parasite through the stratum granulosum and stratum germinativum, the cells in its immediate vicinity are altered. In some cases they appear to be compressed and their nuclei are elongated and hyperchromatic. Other cells, usually only one or two adjacent to the parasite's anterior end, may appear somewhat swollen and have pale turgid nuclei. This condition may precede lysis.

The ferrocyanide technique.—With this technique, the initial local increase in solvent water in the ground substance is manifested by the appearance of a blue color around the site of penetration. This is not as restricted in its distribution as is the Evans blue at a comparable time but is evident well within the

first 15 minutes after the larvae come in contact with the skin. There follows a gradual spread of color down through the dermis and an increase in the intensity of the color until, after 45 to 60 minutes, a maximum is reached. At this point there is a relatively even, intense coloration throughout the dermis and extending into the subcutis. Unlike Evans blue, this persists and the coloration is still found to be maximal in areas exposed to cercariae 2 hours previously (115 minutes before the injection of ferrocyanide). This effect is compared with the Evans blue results in plate II.

Miracidia stained by the Hotchkiss method after Zenker fixation have laterally opening penetration glands, which like those of the cercariae, are filled with granular polysaccharide-containing material. The area described as the gut anlagen is also stained but appears to be more homogeneous. In figure 2 of plate IV, a miracidium is shown in which the ducts of the penetration glands may be seen. Material has been secreted from these and may be seen as a thin line on the unstained surface of the miracidum. Material has also been extruded or secreted from the median structure of this individual and can be seen as a sphere attached to the anterior end of the organism.

In a few instances we were successful in obtaining frozen-dried, Hotchkiss-stained sections of miracidia in the act of penetrating the surface of the snail intermediate host. It is apparent that the glycoprotein membrane in the snail epidermis is altered by this penetration as may be seen in plate IV, figure 1. This is reminiscent of the changes caused by cercariae during passage through the basement membrane of the skin. In the specimen shown, the glands appear to be full of polysaccharide even though the miracidium has been able to pass its anterior end through the snail's surface. In this frozen-dried preparation, there is no evidence of staining of the median gut anlagen mentioned previously. It is interesting to note that unlike the cercariae after they have penetrated the skin, the miracidia after penetrating the snail seem to retain most of the polysaccharide material of their lateral (cephalic) glands.

The glands of miracidia in eggs passing through host tissues are also filled with this Hotchkiss-positive material. In normal eggs this material is not only present within the egg shell but appears to pass through it, forming an exterior coating about the shell. An illustration of this is found in plate V, figure 7. This is particularly well shown in frozen-dried material and is usually not apparent or is less apparent in materials fixed by more usual methods. That such an extrusion of material through the shell occurs has been suggested previously,[22,23] and it has been stated that it aids in passage of these eggs through the tissues. In our infections of mice and rats, we have never seen the flame-like radiating processes described by Hoeppli.[24] The presence of this Hotchkiss-positive material around the egg is characteristic not only of eggs in recently acquired infections but, in some instances, older eggs which have been well isolated by fibrous connective tissue are still alive and producing this substance. Eggs presumed to be dead by virtue of the lack of organization of their contents and eggs produced in uni-

22. Girges, R. 1934, Schistosomiasis (Bilharziasis), London, John Bale and Sons.
23. Faust, E. C. 1946, The diagnosis of Schistosomiasis japonica. II, the diagnostic characteristics of the eggs of the etiologic agent *Schistosoma japonicum*. Am. J. Trop. Med. **26:** 113–123.
24. Hoeppli, R. 1932. Histological observations in experimental Schistosomiasis japonica. China M. J. **XLVI:** 1179–1186.

sexual infections, which again lack viable miracidia, lack the glycoprotein secretion and are readily distinguished due to the absence of this Hotchkiss-positive material (plate V, fig. 6). Eggs passing through the intestinal epithelium and those trapped in the liver are often observed to be associated with alterations in basement membranes and intercellular material in their proximity (plate V. fig. 5 and 7).

In summary, penetration of the skin by cercariae of the two species of schistosomes studied is accompanied by an extensive alteration of extracellular substances in the dermis. The basement membrane beneath the stratum germinativum swells in advance of the penetrating larva, changes its staining characteristics, as shown by the Hotchkiss technique, and apparently disappears. The ground substance also changes locally with respect to its polysaccharide-containing protein as shown by the Hotchkiss technique. A transient increase in color is followed by a loss of reactive groups and a resultant color decrease about the site of the larvae. The changes just summarized take place rapidly, since in many instances an individual organism may penetrate the epidermis and be deep in the dermis within 15 minutes after being placed on the skin surface. Further indications of the type of change taking place and its course are given by the results of the Evans blue technique. As the cercariae penetrate, the immediate site of penetration stains with Evans blue. Stain is bound even before the cercariae completely penetrate the epidermis, i.e., as the basement membrane is being altered, and blue spots may be seen within 2 to 4 minutes after the cercariae are placed on the skin. The production of material which will stain with Evans blue reaches its maximum 15 to 30 minutes after penetration begins and, if Evans blue is injected 30 to 45 minutes after the beginning of penetration, it is obvious that less Evans blue-binding material is present. None can be seen if Evans blue is injected 45 to 60 minutes after cercariae are placed on the skin. Concomitant with these changes in the basement membrane and ground substance there is a local increase in the solvent water which, like Evans blue, is at first sharply localized about the site of the individual penetrating larva. This increase, as shown by the ferrocyanide technique, is again like Evans blue in that it becomes more general. Thus, after 30 minutes penetration the entire dermis in the area being penetrated shows an increase in solvent water. However, we should like to emphasize that unlike the Evans blue, this increase continues until a maximum is reached 45 to 60 minutes after the start of penetration and this maximum persists for hours thereafter.

We interpret these changes in the extracellular material of the dermis as indicating indirectly that enzymes are released by the cercariae during penetration and that their activity is directed against the glycoprotein of basement membrane and ground substance. There is an increase in solubility of these materials at the site of penetration, a change in staining affinity and an increase in solvent water.

It seems likely that a similar mechanism of action is associated with the penetration of the snail by schistosome miracidia and with movement through host tissues of miracidia enclosed within eggs. In both instances, changes are seen in the hosts' Hotchkiss-positive material in the vicinity of the miracidia.

*Strongyloides.*—Both species of *Strongyloides* studied (*S. ratti* and *S. simiae*) cause changes in the skin that differ primarily only in degree from those caused by the penetration of schistosome cercariae.

Larvae placed on the surface of the

skin of an animal previously injected with Evans blue show evidence of their activity within 1 to 1½ minutes. Frequently, blue spots begin to appear as soon as 45 seconds after the larvae are placed on the skin. The maximum number of spots is generally reached within the first 10 to 15 minutes. If enough larvae are present, the spots become confluent and the entire exposed area becomes a solid blue with little or no evidence of the points of individual entry. The Evans blue staining follows the same sequence that was seen in the case of the schistosomes, i.e., when Evans blue is injected after penetration has proceeded for 45 minutes the amount of staining is less than at 30 minutes, and Evans blue injection after 60 to 90 minutes penetration reveals no material in the exposed area able to bind the dye (see plate I, fig. 1 to 4).

A feature of *Stronglyoides* penetration that was not seen with penetration by schistosome cercariae is the formation of a distinct wheal in the area penetrated by larvae. The exposed area first shows small papules which appear within 6 minutes after larvae are placed on the shaved skin. Within 10 minutes after exposure the pebbled appearance of the skin changes to a raised wheal with a distinct border as the papules become confluent. The entire exposed area becomes distinct from the surrounding skin and is blanched. The wheal persists for more than 90 minutes, i.e., long after the Evans blue response is over (plate I, fig. 4). If numbers of larvae greater than those routinely used in these experiments (e.g., 10,000 larvae) are placed on an equivalent area of skin, their penetration will sometimes produce subcutaneous hemorrhage, a type of damage not associated with the routine exposures used in this investigation.

Frozen-dried material sectioned and prepared as previously discussed presents a picture that differs little from that seen following penetration by the schistosomes. However, penetration with *Strongyloides* is somewhat more rapid and production of Evans blue spots prior to the passage of larvae through the basement membrane, as seen with the schistosomes, has not been observed. The localization, spread and disappearance of the products of penetration able to bind Evans blue follow the same pattern as described for the schistosome penetration (plate VI, fig. 1 and 3). Both species of *Strongyloides* pass the basement membrane with ease and may be found well within the dermis 3 minutes after the larvae are placed on the skin. They do not produce the tunnels parallel to the epidermis which are characteristic of the schistosomes.

The changes that take place in the basement membrane and ground substance, as shown by the Hotchkiss technique, are also essentially similar to those described following skin penetration by the schistosomes. Thickening and increase in color of the basement membrane prior to penetration are seldom seen, possibly due to the rapidity of this phase of the alteration. Also, less membrane is affected per larva, i.e., only an area 2 to 3 times the larval diameter loses its characteristic color and apparently disappears. As soon as penetration starts there is an increase in solvent water as shown by the ferrocyanide reaction. This is at first localized very sharply to the immediate area of penetration and after 15 minutes penetration almost exactly parallels the Evans blue response as to its locale and extent (plate VI, fig. 5). Within 30 minutes after larvae are placed on the skin, the entire dermal ground substance of the exposed area is a homogeneous blue green which, in contrast to the Evans blue response, continues to increase in color until a maximum is reached in 1 to 1½ hours. This reaction is

undiminished 3 hours after larvae have been placed on the skin surface, as might be expected since the local wheal and edema persist well past this time.

Both species of *Strongyloides* studied cause changes in the skin that differ only in degree from those caused by the penetration of the schistosome cercariae as shown by the techniques utilized here. In *Section II*, the assumption that some features of the mechanisms of penetration of these two groups are the same is further supported.

*Ancylostoma caninum.*—The filariform larvae of *A. caninum* may be found well within the dermis 7 minutes after they have been placed on the skin surface. Their entrance is not accompanied by the prominent wheal formation characteristic of the *Strongyloides*. Grossly, one can first see evidence of penetration within about 7 minutes in Evans blue-injected rats. As in the other species of helminths studied by this method, maximum color develops within the first 15 to 30 minutes of penetration. If Evans blue is injected after the larvae have been allowed to penetrate for 60 to 90 minutes, there is little or no evidence of change in most instances. Occasionally, a few light spots can be seen but the picture is in no way comparable to that found after the shorter penetration periods. The microscopic findings agree with those described for the other species discussed with regard to Evans blue distribution, changes in Hotchkiss-positive material and increase in solvent water. Following Hotchkiss staining, the basement membrane in some cases can be seen to have disappeared from extensive areas and the color increase of the ground substance is, in some instances, more pronounced with this parasite's penetration than with the other species studied. This is particularly well shown by plate III, fig. 6.

*Nippostrongylus muris.*—The larvae of *N. muris* penetrate the skin fairly rapidly and larvae may be found entering or within the dermis within 15 minutes. No wheal or other gross changes, such as those described for the penetration of skin by *Strongyloides*, are seen. Evans blue injection following skin penetration by this species is without result, i.e., there are no signs of a localization of the dye about the penetrating larvae either grossly (plate I, fig. 1) or microscopically in normal penetration. In 6 to 8-month-old rats, even if larvae are placed on the same area repeatedly every 15 minutes for a period of 2 hours prior to injection of the dye, there is little or no Evans blue staining. The exposed area is a barely discernible pale blue and appears to be slightly thickened by edema without having the appearance of a wheal.* If the Evans blue injection is postponed for 12 to 20 hours after such a multiple exposure, this local edema is more evident and on removal of the skin a pale blue gelatinous bleb is found on the inner surface of the dermis. A similar bleb can be produced by the intradermal injection of an extract prepared from filariform larvae of *N. muris*. This will be discussed further in *Section II*.

The Hotchkiss technique reveals no evidence of color increase of the ground substance with penetration nor is there a definite thickening and increase in color of the basement membrane. Penetration of the basement membrane is

* It should be noted that with the skin penetrating helminths, including *N. muris*, it is possible to produce cutaneous hemorrhage by massive exposure of young (50 to 70 g) rats. Hemorrhagic areas will appear as blue spots following intravenous injection of Evans blue. However, hemorrhage is not produced under the conditions of the experiments reported in this paper, and the Evans blue-binding areas are readily distinguished from those accompanying hemorrhage or traumatic capillary damage.

accomplished without causing relatively large portions of it to be unstained and the exact site of penetration is small and difficult to find unless the larva is seen just as it passes into the dermis.

An increase in solvent water as shown by the ferrocyanide technique does accompany penetration. This is almost identical to that seen in the other forms studied but may be somewhat less pronounced than that occurring with the penetration of the *Strongyloides* and *Ancylostoma*.

To summarize, *N. muris* is unique among the group of skin-penetrating helminths studied in that it does not cause changes in the polysaccharide-containing protein of the basement membrane and ground substance of the type revealed by the Evans blue and Hotchkiss techniques. However, there is an increase in free water, which indicates that some alteration of the ground substance has occurred. This suggests that the mechanism of skin penetration by *N. muris* differs from that of the schistosomes, *Strongyloides* and *Ancylostoma*. This suggestion is supported by the results of later experiments on comparative enzyme activity which are discussed in *Section II*.

*Trichinella spiralis*.—In its infection of the intestine and in the later penetration of muscle fibers by larvae, this organism must pass membranous structures having concentrations of polysaccharide-containing protein. It has been suggested that these larvae possess enzymes used in penetration of muscle fibers.[25] Consequently, the same methods used to study skin penetration by helminths were used to study this organism to see if it, too, caused changes in the basement membranes and other polysaccharide-containing material. The infection was studied in white rats from 3 to 18 months old. The rats were infected via stomach tube with larvae digested free of muscle, the dosage in each instance being 20 larvae per gram of body weight. The hosts were killed at appropriate intervals after being infected.

With the Evans blue technique, some evidence of penetration can be found within 20 minutes after free larvae are introduced into the rat stomach. Blue spots may occasionally be seen at the pyloric end of the stomach and blue villi can be seen scattered throughout the duodenal mucosa. At the end of 2 hours these blue villi are more noticeable and have increased in number. Sections made from the Evans blue material reveal that the blue of the villus is due to an accumulation of dye in the expanded lacteal. There is no evidence of the presence of intensely staining material such as was found in skin penetration. Two hours after infection, the lymphatics of the submucosa as well as those of the tunica propria of the villi retain dye in their lumina. As early as 4 hours, and more noticeably at 16 hours, the local lymph nodes of the intestine and the mesenteric nodes are definitely more blue than in the normal animal. Sections of these show that the most intense color is in the lumen of the peripheral lymphatic channel. In addition, in the peripheral portion of the nodule there is an accumulation of blue granules in the reticular cells. In 24-hour infections, there may be some accumulation of blue-staining material in the vicinity of the developing worm. This cannot be distinguished from the mucoid secretion of the intestine which also stains at this time. In this infection, the Evans blue technique does not reveal changes in the acellular constituents of

25. Jensen, V. and Roth, H. 1938, Zur Einwanderung der Trichinenlarvae in die quergestreifte Muskelfaser. Acta. path. et microbiol. scandinav. suppl. **37**: 259–271.

the intestine comparable to those seen in skin penetration.

Larval stages in the diaphragm were studied in detail in 4-day to 17-month-old infections in the rat. Even in the early stages of infection of the muscle, no evidence of change is apparent in the material prepared by the Evans blue method. During the first 40 days of infection the capillaries of the diaphragm tend to be more engorged with blood and hence retain more dye than normal. In only a few instances can blue spots be seen associated with larvae. Their presence is not consistent and cannot be considered a normal sequelae of muscle fiber penetration unless this stage is so very transient as to be encountered only rarely.

The Hotchkiss technique reveals extensive alterations in the infected muscle. We will include a brief discussion of these changes here although they cannot be ascribed specifically to effects of penetration and are, in later stages, degenerative or reparative host responses to invasion. The newly penetrated muscle fibers, as seen 6 to 20 days after infection, are readily distinguished from their neighboring fibers. They are swollen and the Hotchkiss-positive sarcolemma is somewhat thicker and much more prominent than that of neighboring uninfected fibers. The fiber itself contains a considerable amount of Hotchkiss-positive material so that its nuclei are differentiated from the pink background by their lack of coloration. These features are readily seen in plate V, fig. 1. The area of altered fibers is metachromatic with toluidine blue and corresponds morphologically to the area described by Bullock[26] as showing an increase in alkaline phosphatase. Extensive changes take place with the inflammatory response to the developing trichina. These are somewhat outside the scope of the present study since they represent later host responses which are not directly the result of penetration. However, one extremely interesting feature is the progressive change in the external acellular capsule that forms around the larva. This capsule, which has been described as a thickened sarcolemma, stains distinctly with the Hotchkiss method. With increasing age of infection, the staining of this capsule intensifies as illustrated in plate V, figures 2 and 3, which are from 5 and 10 month infections. When stained with toluidine blue, the capsule is metachromatic and stands out sharply from the surrounding tissue, the basophilic homogeneous debris about the larva, and the larva itself. After 5 months or more of infection the metachromasia is less pronounced although still present. The Hotchkiss staining of the capsule decreases after 10 months and in later stages, when it is obviously laminated in appearance, the intensity of coloration has greatly decreased. The poles of the capsule, which at this time are beginning to calcify, are also much less intensely stained than they are earlier in the infection. A typical example of this later stage is shown in plate V, figure 4. The staining of the material immediately surrounding the larva within the capsule is also less intense than that seen earlier in the infection and is more homogeneous.

*Summary of section I.A*

The Evans blue, Hotchkiss and ferrocyanide techniques were used to study the penetration of the skin by various parasites in order to ascertain whether the acellular constituents of the connective tissue were altered or softened to allow the organisms to pass more freely.

26. Bullock, W. L. 1953, Phosphatases in experimental *Trichinella spiralis* infection in the white rat. Exper. Parasitol. **2**: 150–162.

The infective larvae of the schistosomes, the *Strongyloides* and *Ancylostoma* profoundly change the state of the polysaccharide-containing protein of the basement membrane and ground substance of the skin. At the site of the penetrating larvae, these substances become more soluble and lose their affinity for stain or are removed. There is also an increase in solvent water at the penetration site.

The penetration of the skin by *N. muris* is not accompanied by changes in glycoprotein even though there is a marked increase in the solvent water of the dermal connective tissue. In the case of penetration of the intestinal mucosa by *Trichinella*, no changes in glycoprotein were found that could be considered analogous to the type of change seen in skin penetration. Changes in the muscle fibers of the diaphragm associated with encapsulation of the trichinae are described.

These results are interpreted as indicating that those forms which penetrate the skin elaborate enzymes which aid penetration by altering the resistance of the acellular material to the passage of the larvae. In some of the species studied they act on the polysaccharide-containing protein in such a manner as to suggest that it is depolymerized (schistosomes, *Strongyloides*, *Ancylostoma*), while in others enzymatic activity is suggested only by the fact that there is a local increase in free water in the acellular ground substance at the site of penetration (*Nippostrongylus*).

### SECTION I. B. THE EFFECT OF VARIOUS AGENTS AND HOST CONDITION ON PENETRATION

*The effect of age.*—Age immunity or increased nonspecific resistance with age has been reported for numerous infections, including those with parasitic helminths. The basement membranes and intercellular ground substance of the connective tissue become more highly polymerized with age.[1,7] The following experiments were carried out to ascertain if this increase in polymerization with age results in an inhibition of larval penetration, i.e., in greater resistance of the host basement membrane and ground substance to alteration by larvae or larval products.

Penetration of the skin by *S. ratti*, *S. simiae*, *S. mansoni* and *S. douthitti* was compared directly in young mice and rats (2 to 4 months old) and old mice and rats (18 to 20 months old). The Evans blue method and Hotchkiss technique were used with freezing-drying of the tissues as discussed in the section on methods (section I.A).

The penetration of the skin of rats by *Strongyloides*, *Schistosoma* and *Schistomatium* is slower in old individuals than in young animals, as shown by the time elapsed before the appearance of the first blue spots after an intravenous injection of Evans blue. This is also true of penetration of mouse skin by *Schistosoma* and *Schistosomatium*, though to a lesser extent. When *S. ratti* and *S. simiae* penetrate the skin of young rats, the first spots appear within 45 seconds after larvae are placed on the skin. At the end of 5 minutes many spots can be seen over the exposed area. When older animals are penetrated by these species, the first spots do not appear until 2 or 3 minutes have passed and a condition comparable to the 5-minute exposure of the young animal is not reached in the older ones until about 8 minutes have elapsed. These effects are much more marked and much more consistent in the penetration of the two schistosomes. With these, the first spots appear within 4 to 5 minutes in the young animals and usually do not appear before 10 to 12 minutes in the old animals. In very old rats (over $1\frac{1}{2}$ years), a reaction compara-

ble to the 15-minute response of the young animal is not reached until penetration has proceeded for 25 to 30 minutes. As might be expected, the epidermal layers of the older animals are thicker than those of the younger ones. This does not present any significant barrier to the penetrating schistosomes, as the cercariae may be found to have penetrated all the layers and come to rest on the basement membrane less than 15 minutes after being placed on the skin surface of either young or old animals. However, in old animals, some cercariae can still be seen in this position 24 hours later. In these old animals, cercariae that have been unsuccessful in penetrating to the dermis commonly migrate within the epidermis into the hair follicles or into the sebaceous glands, where they are still excluded from the dermis by the basement membrane. See plate III, figure 5. We have never observed cercariae in these positions in our normal young animals 24 hours after infection. Retention above the basement membrane is not the fate of all cercariae in the older animals as some of them successfully penetrate the membrane in a relatively short time. We have not investigated the fate of those not successful in passing the membrane within 24 hours.

*The effect of hypophysectomy.*—Among the general effects of hypophysectomy in rats is an apparent thickening of the skin. More important from our point of view is the fact that the basement membranes are thickened and stain more deeply. Thus the skin of a young hypophysectomized rat resembles that of a $2\frac{1}{2}$ year-old animal in these respects.[1,7,9] These experiments were carried out in order to ascertain whether or not young hypophysectomized animals with their thickened basement membranes would react to penetration in a manner similar to that seen in old animals which also have a thickened basement membrane, viz., by increasing the penetration time and retarding the progress of the parasite.

Three-month-old rats which had been hypophysectomized 3 weeks previously were utilized in all of the experiments. Control rats were of the same age, strain and sex (females) as the hypophysectomized rats. In most cases the controls weighed about 10% more than the operated animals. The hypophysectomized animals* were exposed for various periods of time to the infective larvae of *S. simiae*, *S. ratti*, *S. mansoni*, or *N. muris*. The methods used were identical to those previously described in *Section I, A* and the histochemical techniques included the Evans blue, Hotchkiss and ferrocyanide methods. At the end of each experiment the rats were examined to determine the success of the original operation and in each instance hypophysectomy was found to be complete.

The gross appearance of areas of the skin of hypophysectomized rats exposed to *S. simiae* or *S. ratti* differs from that of the normal animals in that no wheals are formed and the only evidence of penetration is a barely perceptible local erythema. In the animals injected with Evans blue the first appearance of blue spots is extensively delayed in the hypophysectomized group. In the control animals, fairly large numbers of blue spots appear in 4 minutes and 3 minutes following exposure to *S. simiae* and *S. ratti*, respectively. In the hypophysectomized group only a few spots have appeared at 25 minutes in those exposed to *S. simiae* and only a few appear at 8 minutes in the hypophysectomized animals exposed to *S. ratti*. In hypophy-

* Obtained from Hormone Assay Lab. Inc., Chicago, Ill. These animals were from the same strain as used in all other experiments reported here (originating from the Sprague-Dawley stock and obtained from Holtzman Rat Co.).

sectomized animals exposed to *S. mansoni* this reduction in the number of blue spots is even more apparent and the time of appearance is similarly greater than in the controls. For example, in one experiment the first spot did not appear until 15 minutes after exposure of the skin, at 30 minutes only 2 indistinct blue spots were present, and no more appeared later. In contrast, at 30 minutes the control animal had approximately 200 more or less confluent spots covering the area exposed to cercariae.

The microscopic appearance of the skin of hypophysectomized rats differs from that of control animals of the same age. Following staining by the Hotchkiss method, it can be seen that the basement membrane is very much thicker than that of the controls and stains more intensely (plate VI, fig. 7 and 8). The ground substance is also more intensely stained than in control animals and the epidermis of the hypophysectomized animals is slightly thicker than that of the controls. The general appearance of the skin of an hypophysectomized rat is almost identical to that of an old rat. These features may be seen by comparing figures 6, 7 and 8 of plate VI. Following exposure of hypophysectomized animals to either species of *Strongyloides* there is a greater increase in color of the basement membrane at the site of penetration than in the controls and more larvae are retained in the epidermis and dermis. No great increase in the color of the basement membrane is noted in hypophysectomized animals exposed to cercariae of *S. mansoni* and many larvae are retained in the skin. No cercariae can be found in the dermis 30 minutes after exposure, while in the controls many are seen deep within this layer. Many of the cercariae are still above the basement membrane and excluded from the dermis 90 minutes after exposure of hypophysectomized animals, while in the controls few or none remain in the epidermis at this time. In hypophysectomized animals exposed to *N. muris*, no retention of larvae in the skin was noted.

In sections of the Evans blue preparations from hypophysectomized animals, the ground substance in the dermis is very heavily stained in the immediate vicinity of larvae of *S. ratti*, a change much more intense than that observed in the normal rat (plate VI, fig. 4). In the animals exposed to *S. mansoni*, the normal Evans blue response does not occur and thus no changes can be seen. The sodium ferrocyanide method reveals that the normal increase in free water is delayed in the case of the *S. simiae* penetration, while in the case of *N. muris* penetration it is the same in the hypophysectomized animal as in the control. No increase could be demonstrated in the single *S. ratti* experiment performed by this method.

*The effects of calcium and hyaluronidase.*—Calcium is reported to increase the cohesiveness of the capillary walls by acting on the intercellular cement substance.[27] Hyaluronidase, on the other hand, is reported to have a softening action on the ground substance or to increase capillary permeability. Because of these reported effects it was thought that rats treated with these substances might in the case of calcium exhibit a decrease, or in the case of hyaluronidase show an increase in the rate of penetration of helminth larvae.

Intravenous calcium gluconate (2.5 g/kg) or intravenous testicular hyaluronidase (100 mg of 150 TRU/mg) was administered 30 minutes before larvae were placed on the shaved skin of 200 g rats.

27. Zweifach, B. W. and Chambers, R. 1950, The action of hyaluronidadse extracts on the capillary wall. Ann. N. Y. Acad. Sc. **52**: 1047–1051.

Experiments using *S. ratti*, *S. simiae* and *N. muris* have not given any indication that either of these substances affects the penetration of larvae in the white rat.

*The effect of antihistamines.*—Techniques of intravenous injection of various dyes have been used to study reactions to the intradermal injection of various substances. Such methods are useful for showing increases in capillary permeability induced by the injection of histamine or histamine liberators. After the injection of pontamine blue such injected areas are visible grossly as blue spots and these reactions can be completely inhibited by various antihistamines.[28] Since the Evans blue response found in skin penetration superficially resembles these reactions, experiments were carried out using antihistamines to determine whether or not a similar inhibition might result. Another general similarity between the Evans blue response and the histamine responses studied by Miles and others is the lack of a response if the time between the initial exposure and the dye injection is prolonged. In the case of histamine "blueing," however, an area previously injected becomes refractory or "immune" to further reaction and does not respond to a second injection, while in the case of penetrating larvae where no Evans blue remains after 45 to 90 minutes penetration, a second application of larvae to this area will again result in the production of blue spots (see fig. 5, plate I).

In our experiments with antihistamines, we used 1 to 10 times as much neoantergan as is necessary to completely inhibit the "blued" spots of Miles (0.1 to 1.0 mg/kg intravenous) and even larger amounts of benadryl hydrochloride were used in other experiments (2 and 10 mg/kg intravenous). The antihistamines were administered 5 and 15 minutes before the application of larvae of *S. simiae* and *S. ratti*.

With both of these species the wheal formation noted with normal penetration is reduced following neoantergan injection and in some experiments was almost imperceptible. The increase in free water is less than in the controls exposed for the same period of time. However, the time of appearance and the intensity of the Evans blue spots produced by the penetrating larvae are not affected. In fact, injection of the dye in less that 1/10th the usual amount still results in excellent localization of the site penetrated. Both of the antihistamines cause a blanching of the skin but the benadryl causes only a slight decrease in the wheal formed.

*The effect of cortisone.*—It is now general knowledge that cortisone and related steroids profoundly affect the state of the connective tissue. It was thought that large doses of cortisone acetate might alter the normal response of the ground substance to penetration by helminth larvae. For this reason, 3-month-old white rats were given 25 mg of cortisone subcutaneously daily for a total of 5 days. On the 5th day, 2 hours after the last injection of cortisone, the treated animals and controls were exposed to infective larvae of *S. ratti* and observations were made using the Evans blue and Hotchkiss techniques. The treated animals appeared to have a thinner skin due to a loss of fat from the subcutis and were about 12 g lighter (110 g) than the control animals of the same age group.

In normal animals, minute papules can be seen in the shaved area being penetrated by *Strongyloides* within 2 or 3 minutes after exposure to the larvae.

28. Miles, A. A. and Miles, E. M. 1952, Vascular reactions to histamine liberator and leukotaxine in the skin of guinea pigs. J. Physiol. **118**: 228–257.

Their appearance precedes the actual wheal formation which involves the entire exposed area. In the cortisone-treated animals these small papules do not appear though it is obvious from the twitching movement of the cutaneous muscles that penetration is taking place. Eight minutes after larvae are placed on the controls, the exposed area is pebbled with minute wheals or papules which become confluent by 14 minutes. No such change can be seen in the cortisone-treated animals. The Evans blue response is distinctly different in the 2 groups. The first spot appears in 8 minutes in the cortisone-treated animals and at 1½ minutes in the controls. Only a few spots can be seen in the cortisone-treated group at 15 minutes and at 30 minutes the cortisone group has approximately the same amount of response as is found in 15 minutes in the control group. It should be noted that the response is not exactly identical to that usually seen as the blue spots produced in the cortisone-treated animals appear smaller and do not have as extensive a diffuse blue halo as do the spots in the normal animals.

Sections from the Evans blue material were examined as well as sections prepared by the Hotchkiss and the ferrocyanide techniques. The areas of dye retention in the Evans blue sections differ somewhat from those usually seen. Staining is more intense and relatively sharply localized about the larvae, and in some instances extends up into the epidermis in a manner similar to that seen in immune animals (plate VI, fig. 2). Another marked difference between the treated and control animals is the retention of some larvae in the dermis of the cortisone-treated group 30 and 60 minutes after the start of penetration. In normal animals we seldom find larvae of this species in the dermis at 30 minutes and almost never see them at 60 minutes. With the ferrocyanide technique the cortisone animals showed somewhat less color than the controls at 1 hour but this was not a striking difference in the single series of animals used.

*The effects of immunity.*—Helminths that penetrate the skin are often immobilized in the dermis in immune animals and inhibited from completing their cycles. The following experiments were conducted to determine whether or not host immunity would prevent penetrating larvae from causing changes in the basement membrane and ground substance and to ascertain whether any local immunity in the skin could be revealed by the Evans blue and Hotchkiss techniques.

Using the Evans blue technique, a comparison has been made of normal rats, rats immunized by repeated subcutaneous injection of larvae of *S. ratti* in the inguinal area and rats immunized by repeated exposure of a single small area of the skin to penetrating larvae of *S. ratti*. Rats of the same age, weight, sex and strain were used for all 3 groups and animals from each group were tested simultaneously using the same pool of larvae. The rats immunized by subcutaneous injection were infected every 2 weeks, receiving an initial infection of 2000 larvae followed by 3 to 5 infections of 5000 larvae each. The rats immunized by allowing larvae to penetrate the skin were exposed in a single middorsal area just posterior to the scapulae. The area was approximately 2 cm in diameter and its outline was tattooed so that the exact area could be repeatedly exposed. This group was exposed to 2000 larvae initially, followed by 3 or 4 exposures to 3000 or 5000 larvae at 2 week intervals. For the final test infection, each area was exposed to 10,000 larvae. In one experiment, the locally immunized area was compared

with other areas on the same animal, with similar areas on the animals immunized by injection of larvae and with nonimmune animals. The rats at the time of this experiment were 200 to 220 g in weight. As in previous experiments the animals were studied using the Evans blue and Hotchkiss methods.

Rats immunized by several previous exposures to *S. ratti* have an extensive skin reaction to penetrating larvae. A large wheal forms and there is obvious erythema. In addition, the exposed area "weeps" and subcutaneous hemorrhage may occur within a few minutes after exposure. Penetration in the immunized animals is apparently somewhat slower than in the control animals though it is not prevented. Whereas the first Evans blue spots appear in the nonimmune controls in less than a minute, they usually do not appear in the immunized hosts for 5 to 7 minutes. In another similar experiment they appeared in 3 minutes in the immunized animals. The most intense blue reactions are found in the immunized animals. In the animals immunized by normal penetration of larvae, the specific area previously exposed shows a greater reaction than the adjacent area. In these specific areas blue is still retained 90 minutes after the test exposure. This may well be due to the increased hemorrhage in these areas. Surprisingly enough, in the animals immunized by repeated penetration of larvae through one area of the skin, the adjacent areas previously unexposed react more like areas on nonimmune animals than like those on the animals immunized by injection, i.e., the wheals are as small as those found in the normal animals and no Evans blue spots remain at 90 minutes. However, the first appearance of spots in these adjacent areas is retarded just as in the other areas of both immune groups. Sections of the skin of animals from experiments identical to the above were examined. In tissues taken 2 weeks after the last of a series of penetrations and stained by the Hotchkiss method, there is no evidence that any change has taken place in the basement membrane and ground substance with respect to their glycoprotein. There is a considerable increase in hair growth on these areas of repeated exposure. Many of the changes which take place subsequent to the application of larvae on the skin of these immune animals are well shown by the use of the previously discussed histochemical techniques. However, these will not be elaborated here as they more properly belong to another investigation.

*Summary of section I. B*

Skin penetration by *Strongyloides* and the schistosomes is slower in old animals than in young mature animals. In many instances, the thicker basement membrane of the old animals acts as a barrier to penetration so that schistosome larvae which might pass through the membrane of a younger animal in 15 minutes are excluded from the dermis for as long as 24 hours. Even more marked inhibition of penetration is found in hypophysectomized rats. These have basement membranes that are thickened and, in general, resemble those of very old animals with respect to the organization of the glycoprotein constituents of the skin. The Evans blue response in these animals is greatly delayed or completely inhibited, as in the case of the schistosomes. Many larvae are unable to pass the basement membrane or are retained in the dermis. Wheal formation is absent. With the exception of *N. muris*, free water is less than in the controls. Experiments with calcium-treated and hyaluronidase-treated animals revealed no differences from the normal in the ability of the organisms to penetrate or in the rate of penetration. Antihistamines do not retard penetration nor do

they influence the development of the Evans blue response to penetrating larvae. In animals treated with these substances, there is a slight decrease in wheal formation following *Strongyloides* penetration and less increase than usual in the amount of free water in the dermis. Animals treated with cortisone differ markedly in their response to penetrating *Strongyloides*. The Evans blue response is delayed, more larvae are retained in the dermis, wheal formation is absent and the amount of free water is less than in normal animals following penetration. Skin penetration is also inhibited in immune animals and many larvae are retained in the dermis. The Evans blue response is very intense but is delayed in appearance. These effects are most noticeable in the specific areas of skin repeatedly exposed to larvae during immunization.

## SECTION II. ENZYMATIC ACTIVITY OF LARVAE IN VITRO AND IN VIVO

Various tests for collagenase, proteolytic activity and "spreading factor" were performed with living larvae, homogenates and extracts of infective stages of a number of nematode and trematode parasites and with extracts of larvae and adults of one nonpenetrating nematode.

### *Preparation of material*

Filariform larvae of *S. simiae*, *S. ratti*, *N. muris* and *A. caninum* were obtained from charcoal cultures of feces and concentrated with a modified Baermann apparatus. This modification permitted relatively rapid (10 minute) initial concentration of the larvae in a small volume (250 ml) without the inclusion of debris in the concentrate. The apparatus consisted of two layers of closely woven unbleached muslin within a 5-inch sieve, placed in water at 39 C in a shallow pyrex dish on a warming plate regulated at 40 C. Cercariae of *S. mansoni*, *S. douthitti* and of a strigeid, and several types of stylet cercariae were collected after normal emergence from the snail host or were dissected free of infected snails which had been carefully cleaned beforehand. Larvae and adults of *Rhabditis pellio* were cultured on maltose agar plates and recovered from the surface by rinsing with distilled water. Larvae of *T. spiralis* were digested free of rat muscle with a mixture of pepsin and hydrochloric acid.

The larvae thus obtained were concentrated by centrifuging and washed a minimum of 4 times by resuspending in 80 ml of distilled water and recentrifuging. In a number of experiments, the larvae were washed and concentrated in distilled water containing 1000 units of penicillin and 1 mg streptomycin per ml. Homogenates were prepared by grinding the larvae in a glass homogenizer in an ice bath and then centrifuging at approximately 600 RCF for 30 minutes. The opalescent supernatant fluid obtained was used as the test material. These homogenates were prepared in such a way that each ml was derived from about $10^5$ larvae. Extracts of larvae were made in a slightly different manner. The thoroughly washed concentrated larvae were frozen and lyophilized at −30 C and then weighed and ground with a small volume of cold sterile saline (0.85%) in a glass homogenizer immersed in an ice bath. The resulting material was centrifuged at 4 C at 18,000 RCF for 1 hour and the clear supernatant extract was collected. Each ml of extract was derived from approximately $2\times10^6$ larvae. The series of extracts used for comparison of extract activity on the basis of dry weight (table 2) were further treated by passing them through a micro Seitz filter into sterile tubes.

### *Tests for collagenase activity*

In view of the fact that changes in the basement membrane and ground substance are often accompanied by the presence of collagenase activity,[1] extensive tests for collagenase activity were performed using extracts, homogenates and living organisms. The helminth larvae selected included not only those which had been shown by the histochemical study to produce extensive changes in these acellular substances but also forms unable to cause these changes. An azo dye-coupled hide powder collagen (azocoll)* was used in these tests in the manner described by Oakley

* For this preparation of azocoll we are indebted to Dr. H. R. Catchpole of the University of Illinois Medical School, Department of Pathology.

et al[29,30] for determination of collagenase activity of *Clostridium welchii*. The amount of dye freed after a stated period of incubation with *Cl. welchii* toxin can be correlated with the collagenase activity of the toxin.

The extracts used in the azocoll tests as well as materials removed from the azocoll system after testing were checked for sterility using an enriched thioglycollate medium and in the occasional instance where contaminants were found, the contaminating organisms were also checked for collagenase activity under the same conditions as used in the experiment with the extract. In the experiments reported, none of the occasional contaminants recovered were active against the azocoll substrate under these conditions.

As a positive control, 6 mg of lyophilized *Cl. welchii* toxin (of which 80 to 90% was dextrose added prior to drying) were used. This is an arbitrary amount chosen for the convenience of the experiment. This amount of the particular lot of toxin used freed enough dye in the azocoll-buffer system to be definitely perceptible to the eye (+) within 2 hours and strongly positive (++) within 4 hours, while after 16 hours all dye was freed from the azocoll substrate (++++). Various other controls such as buffer controls, inactivation by heat, host feces and contaminants from cultures were also included where applicable to the experiments.

Material to be tested was incubated at 37 C with 6 mg of azocoll in $M/10$ phosphate buffer, in most cases at pH 6.5. In each test the total volume was 1 ml, of which 0.5 ml was the homogenate, extract or larval suspension being tested. This included 1000 units of penicillin and 1 mg of streptomycin. The reaction was carried out in stoppered 10×75 mm pyrex tubes which were examined at the end of 1, 2, 4, 16 and 20 hours of incubation. The amount of dye released is expressed by ±, +, ++, +++ or ++++, as designated for the toxin control. A ± value indicates a color increase barely perceptible to the eye and is comparable to that observed occasionally in a buffer control after 20 hours incubation. Even though the tests were carefully designed to rule out the

29. Oakley, C. L., Warrack, G. H. and Van Heyningen, W. E. 1946. The collagenase (k-toxin) of *Cl. welchii* type A. J. Path. & Bact. **58:** 229–253.

30. Bidwell, E. and Van Heyningen, W. E. 1948, The k-toxin (collagenase) of *Clostridium welchii*. Biochem. J. **42:** 140–151.

PLATE IV

Penetration glands of cercariae and miracidia as shown by the Hotchkiss technique.

FIG. 1.—Miracidium of *S. mansoni* penetrating snail (*Australorbis*). The glycoprotein of the snail epidermis is altered in the vicinity of the miracidium (m). The laterally opening penetration glands of the miracidium can be seen in the anterior end of the organism just above the arrow (a). Frozen-dried material, 10 $\mu$, ×350.

FIG. 2.—Miracidium showing lateral penetration glands (cephalic glands) and ball of exudate at anterior end. Zenker-fixed smear, ×470.

FIG. 3.—Cercarial body of *S. douthitti* removed from sporocyst. The penetration glands with granular Hotchkiss-positive contents may be seen (c) as well as the ducts. Nuclei of the glands (n) are unstained. Smear fixed in absolute alcohol, ×350.

FIG. 4.—Newly emerged *S. douthitti* cercaria showing considerable decrease in gland content as compared to fig. 3. Only three ducts can be clearly seen. Smear fixed in absolute alcohol, ×350.

FIG. 5.—*S. douthitti* cercaria on mouse epidermis showing loss of most of gland contents. One of a group of serial 15 $\mu$ sections from frozen-dried material, ×630.

FIG. 6.—Anterior end of cercaria illustrated above (fig. 3) to show detail of polysaccharide-filled ducts. At (d) 6 may be seen. ×770.

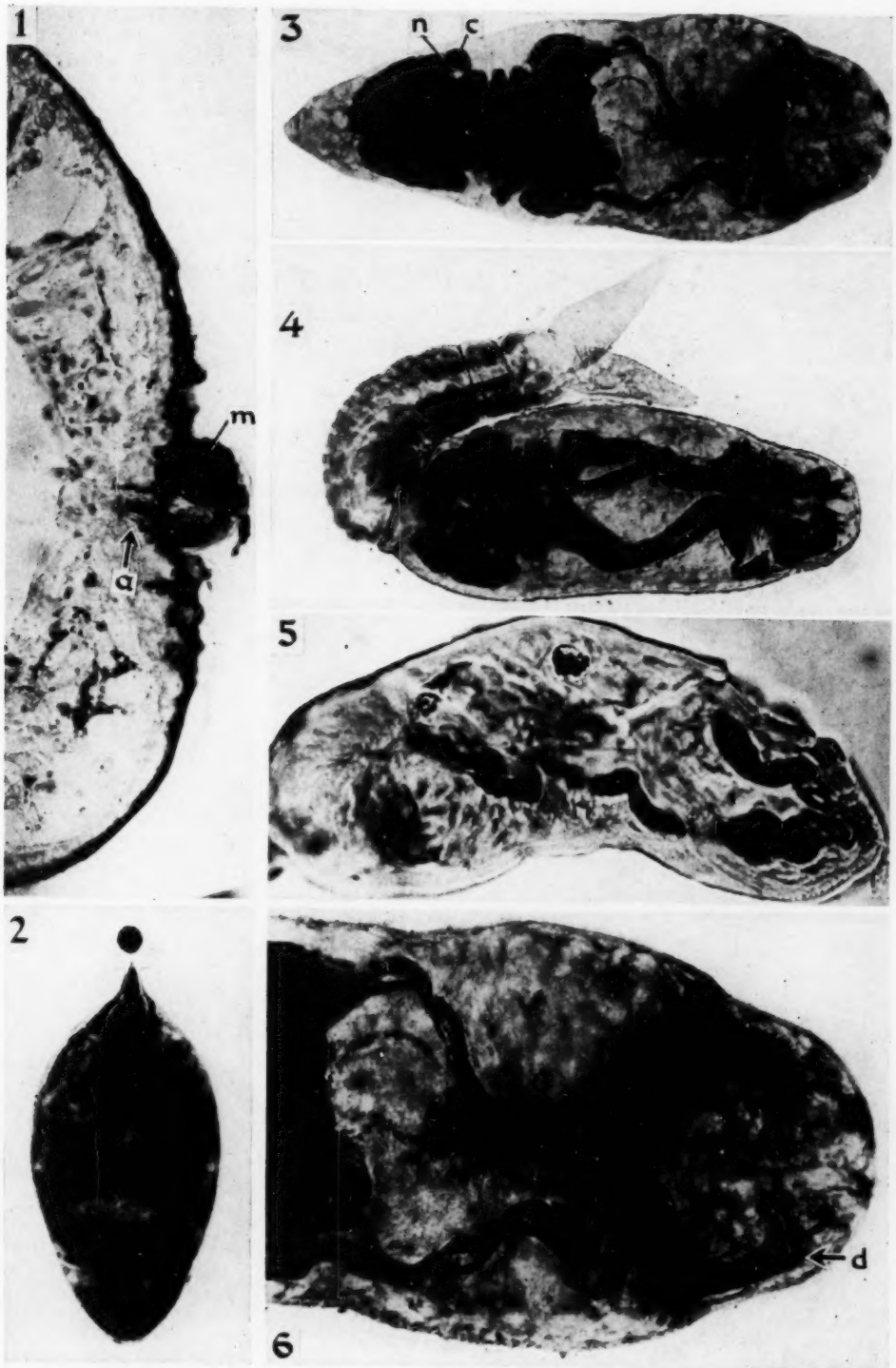



PLATE IV

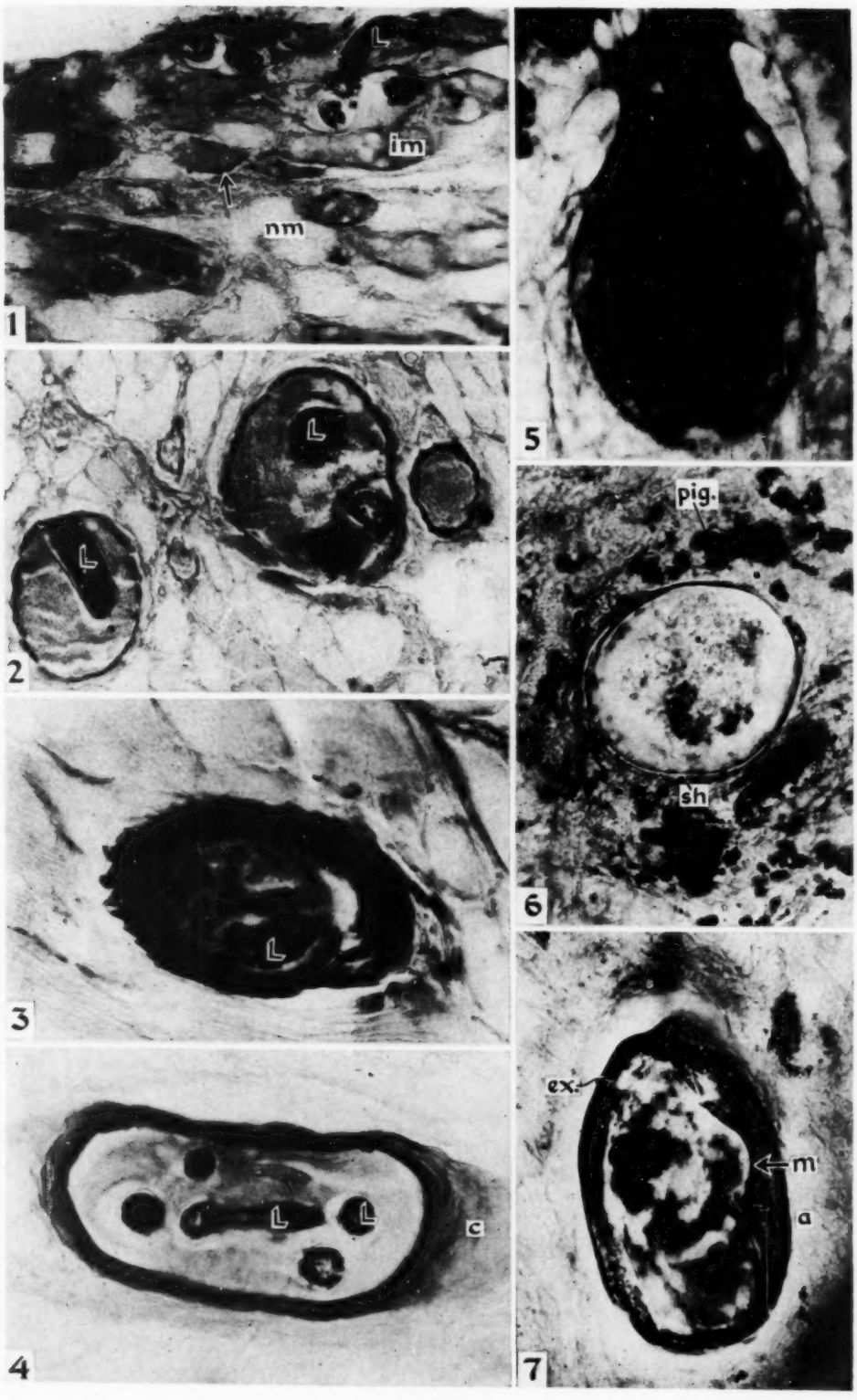



PLATE V

action of possible contaminants through the use of controls and the addition of antibiotics, only those consistently showing dye release within 4 hours incubation were considered unequivocally to be due to the action of the larvae. The results of the various tests are summarized in tables 1 and 2.

Cercariae of both *S. mansoni* and *S. douthitti* were positive for collagenase within 4 hours when as few as 300 cercariae were used. Even larger numbers of the other types of cercariae tested uniformly gave negative results.

Living larvae of *S. simiae*, *S. ratti* and *A. caninum*, as well as extracts of the first two species, gave positive results and were almost instantly positive when concentrated extracts were used. The equivocal findings with the *A. caninum* extract may possibly be due to the fact that the larvae were obtained from 3-month-old cultures. Material from *N. muris*, *T. spiralis* and *R. pellio* gave consistently negative results even when an extract of as many as $1.6 \times 10^6$ larvae was used in the test (*N. muris*, table 2). These were also negative at pH ranges other than that used routinely.

### *Other tests for proteolytic activity*

Three techniques, namely digestion of thin gelatin films, digestion of tissue sections and the Anson test[31] were adapted for the purpose of screening the various helminth larvae for proteolytic activity.

*Digestion of gelatin.*—Living larvae and larval extracts of *S. mansoni*, *S. ratti*, *S. simiae*, *N. muris*, *R. pellio* and

31. Anson, M. L. 1938, The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. **22**: 79–89.

←

PLATE V

Various stages of helminth larvae in tissues stained by the Hotchkiss method.

FIG. 1–4.—*T. spiralis* larvae in rat diaphragm showing alteration and accumulation of glycoprotein about larvae at various periods. Frozen-dried, 15 μ paraffin sections, ×210.

FIG. 1.—Twenty-day infection: The larvae (L) contain much polysaccharide and the individual infected muscle fibers (im) are more intensely stained than the uninfected fibers (nm). The sarcolemma of the infected fibers is thickened and more intensely stained as indicated by arrow.

FIG. 2.—Five-month infection: The sarcolemma of the fibers throughout the diaphragm is more intensely stained than normal and stands out as a network. The larvae stain heavily and are surrounded by a mass of glycoprotein. The relatively thin capsule separating them from the muscle is intensely stained and is not obviously laminated.

FIG. 3.—Ten-month infection: The larva is surrounded by glycoprotein which has become more homogeneous. The capsule is thicker, laminated and very intensely stained.

FIG. 4.—Seventeen-month infection: The intensity of the reaction of the glycoprotein surrounding the larvae has decreased and the material is homogeneous. The laminated capsule is much less intensely stained than in the previous example. At the poles of the capsule as at (c), the decrease in staining intensity is most marked. At these sites calcification has started. (Calcification of the capsule poles of this larva was confirmed by staining adjacent serial sections with Alizarin Red S.)

FIG. 5–7.—Schistosome eggs in tissues, frozen-dried, 15 μ paraffin sections, ×625.

FIG. 5.—Viable *S. mansoni* egg in hamster intestine. The polysaccharide-containing secretion of the egg is so intensely stained that the egg is almost opaque. The acellular material about the egg is more intensely stained than that in normal tissue nearby.

FIG. 6.—Infertile *S. douthitti* egg from unisexual infection, in mouse liver. The egg content is unorganized and contains little Hotchkiss-staining material. The shell (sh) lacks the glycoprotein secretion seen in viable eggs.

FIG. 7.—Normal *S. douthitti* egg in mouse liver showing secretion covering inner and outer shell surfaces (ex), border of miracidium (m) with heavily stained material, and area of decreased staining (a) surrounding egg.

TABLE 1.—*Collagenase activity of helminth larvae* incubated with azocoll.†*

| Material tested | Approximate number of larvae | Hours incubated at 37 C | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 4 | 16 | 20 |
| *Cl. welchii* toxin | (6 mg) | – | + | ++ | ++++ | ++++ |
| *S. mansoni*, living | 300 | – | – | + | ++ | |
| *S. douthitti*, living | 500 | – | ± | + | ++ | |
| Plagiorchid, living | 2,000 | – | – | – | ± | |
| Strigeid, living | 2,000 | – | – | ± | ± | |
| *S. simiae*, | | | | | | |
| living | 100,000 | – | – | + | ++ | +++ |
| homogenate | 200,000 | + | ++ | +++ | ++++ | |
| extract | 200,000 | + | + | ++ | +++ | ++++ |
| supernatant fluid‡ | 100,000 | – | – | + | ++ | ++ |
| *S. ratti*, | | | | | | |
| living | 100,000 | – | ± | + | +++ | |
| homogenate | 100,000 | – | ± | + | ++ | |
| extract | 200,000 | + | + | ++ | +++ | |
| *A. caninum*, | | | | | | |
| living | 1,000 | – | – | – | +++ | |
| | 3,000 | – | + | ++ | +++ | |
| homogenate | 1,000 | – | – | – | + | |
| extract‖ | 200,000 | – | – | ± | + | |
| *N. muris*, | | | | | | |
| living | 100,000 | – | – | ± | ± | |
| homogenate | 50,000 | – | – | – | ± | ± |
| | 500,000 | – | – | – | – | – |
| extract | 100,000 | – | – | – | – | – |
| *T. spiralis*, | | | | | | |
| living | 35,000 | – | – | – | ++ | |
| homogenate | 100,000 | – | – | ± | ++ | |
| extract | 2,000 | – | – | ± | + | |
| *R. pellio*, | | | | | | |
| living | 8,000 | – | – | – | – | |
| homogenate | 8,000 | – | – | – | – | |
| extract | 25,000 | – | – | – | ± | ± |
| Phosphate buffer | | – | – | – | – | ± |
| Snail feces | (1:50) | – | – | – | ± | |
| Rat feces | (1:5000) | – | – | – | – | – |
| Monkey feces | (1:5000) | – | – | – | – | ++ |
| Dog feces | (1:5000) | – | – | – | – | – |

* All larvae tested are infective forms, except in the case of *R. pellio*, where both larvae and adults were used.
† Six mg of azocoll, 1000 units of penicillin and 1 mg of streptomycin per tube. All buffered with phosphate buffer at pH 6.5 in a total volume of 1 ml.
‡ From 100,000 washed larvae placed in ½ ml of distilled water at room temperature for 2 hours.
‖ Prepared from 3-month-old culture.

*T. spiralis* were tested for ability to digest a thin gelatin film. The method used by the authors was essentially the same as that described by Pickford and Dorris,[32] who made use of the gelatin films of lantern slide plates as a substrate. Thinner films prepared in our laboratory were also used. These were prepared by dipping microscope slides in a 5% solution of Eastman purified pig-skin gelatin and allowing them to drain and dry in a vertical position. A $1 \times 10^{-6}$ solution of Crystalline Trypsin (Worthington) was used as the positive control and phosphate buffer (pH 7.0 to 7.4), saline or distilled water was used as negative control. Small drops (0.01 ml) of test materials or control fluids, buffered as well as unbuffered, were placed on the plates and allowed to stand at 20 C for 2 hours. At the end of this time the plates were fixed in 4% formalin for 10 minutes, washed with water and then stained with Delafield's hematoxylin for 5 minutes. Enzymatic activity was evidenced by the presence of lighter areas on the stained film where digestion had taken place.

This experiment demonstrates gelatinolytic activity in the living larvae and larval extracts of *S. mansoni*, *S. ratti* and *S. simiae* but not in those of *N. muris*, *T. spiralis* and *R. pellio* (see plate I, fig. 6). However, since one test drop usually contains less than 100 living larvae while a drop of extract represents at least 1000 larvae, positive results obtained with the living larvae are much weaker than those obtained with extracts.

32. Pickford, G. E. and Dorris, F. 1934, Micromethods for the detection of proteases and amylases. Science, **80**: 317–319.

*Digestion of tissue sections.*—Extracts of filariform larvae of *S. simiae*, *S. ratti* and *N. muris* and of larvae and adults of *R. pellio* (those used in the azocoll test shown in table 2) were tested against sections of normal rat skin. The skin was prepared by the freezing-drying method and was sectioned 15 $\mu$ thick. After fixation in absolute alcohol and hydration, sections on the slides were covered with extracts buffered at pH 7.4 and placed in a moist chamber. They were incubated at 37 C for periods of time up to 8 hours and then stained by the Hotchkiss technique.

Sections of skin exposed to extracts of *S. simiae* and *S. ratti* have been almost completely digested in most of the trials. In those exposed to less concentrated extract and in those incubated less than 8 hours, there are decreases in the degree of staining of the basement membrane and the ground substance as compared to those exposed to buffer only. Extracts of *N. muris* and *R. pellio* are inactive, as is the buffer.

*Anson test for trypsin.*—Homogenates of *S. simiae*, *N. muris*, *T. spiralis* and *R. pellio* were tested for "tryptic" activity using the Anson[31] technique. None showed any such activity.

*Tests for "spreading factor"*

Ginetsinskaya[14] reported that intradermal injection of extracts prepared from two stylet cercariae (*C. gracilis* and *C. pseudarmata*) mixed with dye (indigo-carmine) resulted in definite spreading of the dye within 15 minutes. Since the completion of our experiments, "spreading" has been reported following the injection of a homogenate of cercariae of *S. mansoni*[13] accompanied by Evans blue in the inoculum. In our tests for "spreading" activity, 1% Evans blue in saline (1 ml per 100 g weight of rat or rabbit) was given intravenously immediately following intradermal injection of 0.01 ml of homogenate, extract, hyaluronidase, saline or water, or alternatively, the substance being tested was mixed with 3% Evans blue in saline and 0.01 ml injected intradermally.

Up to the present time we have tested *N. muris*, *S. ratti*, *S. simiae*, *R. pellio*, *T. spiralis*, *S. mansoni* and two stylet cercariae. Within 1 hour after injection all of these cause some "spreading" of the dye and with the intravenous injection of the dye, all cause the concentration of dye in the dermis. The areas so affected are much greater in extent than either the saline or water control. How-

TABLE 2.—*Collagenase activity of various nematode extracts compared on the basis of dry weight.**

| Nematode | Dry weight in mg | pH of phosphate buffer | Hours of incubation at 37 C | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 4 | 16 | 20 |
| *N. muris* | | | | | | | |
| filariform larvae | 25 | 6.5 | − | − | − | ± | ± |
| | 5‖ | 6.5 | − | − | − | − | ± |
| | 5† | 6.5 | − | − | − | − | − |
| | 45 | 5.2 | − | − | − | − | − |
| | 45 | 7.9 | − | − | − | − | − |
| *R. pellio* | | | | | | | |
| larvae and adults | 5 | 6.5 | − | − | − | − | ± |
| | 5† | 6.5 | − | − | − | − | − |
| *S. ratti* | | | | | | | |
| filariform larvae | 5¶ | 6.5 | + | + | ++ | +++ | ++++ |
| | 5 | 7.9 | − | − | ± | + | + |
| | 5† | 6.5 | − | − | − | − | − |
| *S. simiae* | | | | | | | |
| filariform larvae | 5 | 6.5 | ++‡ | +++ | +++ | ++++ | ++++ |
| | 5† | 6.5 | − | − | − | − | − |
| *A. caninum* | | | | | | | |
| filariform larvae | 10 | 6.5 | − | − | ± | + | + |

* Incubated with 6 mg of azocoll, 1000 units of penicillin and 1 mg of streptomycin in a total volume of 1 ml.
† Extracts heated to 60 C in water bath for 5 minutes.
‡ Color change was perceptible in 10 minutes.
‖ Approximately 100,000 larvae.
¶ Approximately 200,000 larvae.

ever, the "spreading" is not the same as that produced by the hyaluronidase control. For example, in the case of *N. muris*, within 1 hour following injection a thickened, gelatinous bulla-like area is produced in which the dye has spread or is concentrated depending on the method used. This is several times the diameter of the control area and on removal of the skin from the test animal, this gelatinous mass can be seen clinging to the skin's inner surface. Material from the other helminths tested gives results similar to that obtained with *N. muris* but somewhat lesser in extent. A single test was performed to attempt to determine hyaluronidase activity of extracts of *N. muris*, *S. ratti*, *S. simiae* and *R. pellio* by a turbidimetric method.* No evidence of activity could be demonstrated.

### *Summary of section II*

Incubation with azocoll consistently reveals collagenase activity in living larvae of *S. ratti*, *S. simiae*, *S. mansoni*, *S. douthitti* and *A. caninum* and also in extracts of the first two nematodes. The results are equivocal with *A. caninum* larval extract and uniformly negative with larvae of *T. spiralis* and *N. muris* and with larvae and adults of *R. pellio*.

Gelatinolytic activity and ability to digest tissue sections is demonstrable in larvae of *S. ratti*, *S. simiae* and *S. mansoni* but not in those of *N. muris*, *T. spiralis* and *R. pellio*.

Intradermal injection of larval extracts of *S. ratti*, *S. simiae*, *N. muris*, *T. spiralis*, *S. mansoni*, *R. pellio* or unidentified stylet cercariae, followed by intravenous injection of Evans blue, results in a concentration of dye in the injected area and produces some "spreading" but this is different in nature and degree from that produced by hyaluronidase.

## DISCUSSION

The results obtained from the histochemical portion of these investigations demonstrate that the movement of helminth infective larvae through the skin and other tissues imposes extensive alterations on the basement membrane and ground substance of the hosts' connective tissue. These changes can be correlated with enzymatic activity of those helminths capable of causing such alterations.

The three techniques that have proved to be of most value in the histochemical portion of this investigation are the Hotchkiss method, the intravenous Evans blue technique, and the method we have designated as the ferrocyanide technique. These have been developed by investigators studying the state and changes of state of the extracellular elements of connective tissue with particular emphasis on the basement membrane and ground substance. Because the state of organization of these substances is associated with the relative amounts of soluble materials, the freezing-drying method of tissue preparation is an indispensable adjunct. The first portions of the discussion are based on our findings using these methods and for that reason a brief summary of their significance and interpretation is included at this point. These interpretations are based on the investigations of Gersh, Catchpole, and their coworkers and have previously been cited.

The Hotchkiss technique when used with proper controls demonstrates polysaccharide-containing protein. The intensity of staining is believed to be related to the number of reactive groups or to the amount of material present and also to the degree of association or dissociation of the material. Up to a point,

* For this test we are indebted to Dr. Albert Dorfman, Department of Pediatrics, University of Chicago School of Medicine.

more intense staining indicates more polysaccharide and, thus, the presence of more glycoprotein. However, it has been shown that highly polymerized glycoprotein may give a less intense staining reaction. In the latter case, the reaction of highly polymerized glycoprotein may be increased following the exposure to enzymes which presumably depolymerize it and allow more reactive groups to be exposed, thus giving a more highly colored product with the reagent. Enzymes may cause an even greater alteration of the glycoprotein material. This may result in the production of soluble products with the loss of reactive groups and a decrease in the intensity of the staining when this material is removed. This condition may be brought about by the action of "mucase system" enzymes.

In connective tissues where glycoprotein substances are changing to a more soluble or depolymerized state, the sites of such activity preferentially bind Evans blue dye when the dye is introduced intravenously in the living animal. This localization of change correlates very well with the changes observed with the Hotchkiss method and is considered to be almost diagnostic of the presence of water-soluble glycoprotein.

The ferrocyanide method has not been used as extensively as have the techniques just described. The findings with this method are interpreted as showing the presence of extracellular free water or solvent water. The capillaries are presumably freely permeable to the ferrocyanide ion and an extracellular accumulation beyond normal may be visualized as a color increase in an area imbibing or freeing water. Areas of this type in the ground substance can be correlated with areas in which the glycoprotein material is being altered to a more soluble state. Because of this, the same areas which show changes from the normal with the Hotchkiss and Evans blue techniques may also show an increase in free water.

The parasites causing the most well defined changes of these acellular constituents include schistosome cercariae and the infective larvae of *Strongyloides* and *Ancylostoma*.

In the normal animal, the penetration of the schistosomes can be considered more or less typical of the group as far as the mechanisms of penetration are concerned although they do differ from the others in some minor details that will be mentioned later. The epidermis is penetrated rapidly and the cercariae (plate III). It seems probable that nativum and come to rest on the basement membrane. Having reached this point, they may progress along the membrane surface for a short distance before further penetration. During the period just described there appears to be some lysis of epidermal cells and a secretion of Hotchkiss-positive material from the penetration glands of the cercariae (plate III). It seems probable that this release of polysaccharide-containing protein is related to the secretion of enzymes aiding in penetration. However, there is no direct evidence to that effect. The mucoid secretions of a variety of cercariae have been intensively studied by Kruidenier,[33,34] who attributes to them a protective role, in the sense that a coating of mucoid material may prevent damage to the cercariae by the host or may protect the organism from its own enzymes. In the case of the schis-

33. Kruidenier, F. J. 1951, The formation and function of mucoids in virgulate cercariae including a study of the virgula organ. Am. Midl. Nat. **46**: 660–683.

34. Kruidenier, F. J. 1953. Studies on the formation and function of mucoids in cercariae: Non-virgulate Xiphidiocercariae. Am. Midl. Nat. **50**: 382–396.

tosomes, the glycoprotein of the penetration glands is not applied to the cercarial surface as a protective coating as seen in some of the forms studied by Kruidenier but rather is applied to the surface of the host. This does not necessarily rule out an "anti-enzymatic" function. It is not outside the realm of possibility that this viscous material acts as a carrier for the enzyme, inhibits its spread and thus confines its activity to the site being penetrated. Another interesting speculation by Oliver-González and González[35] assigns a protective function to polysaccharide secreted by cercariae. This secretion has been demonstrated by immunological methods and it contains an immunologically active polysaccharide. It is similar to a blood group polysaccharide and acts as an $A_2$ agglutinogen.

Within a very few minutes after the cercaria has reached the basement membrane, the portion of the membrane on which it rests becomes slightly swollen and shows evidence of depolymerization. Simultaneously, there is a local increase in soluble glycoprotein and free water in the ground substance at a point immediately beneath the larva. Ground substance changes begin to occur even before the basement membrane is completely penetrated. As the larva passes into the dermis, a portion of the basement membrane can no longer be visualized, i.e., it appears to have been dissolved or altered in such a way that its glycoprotein no longer reacts to the Hotchkiss staining method. During this period there is an extensive increase in the water-soluble material and free water of the ground substance. Within 40 to 60 minutes after the start of penetration, the situation with respect to the water-soluble glycoprotein is different from that at the earlier periods. Evans blue is no longer localized about the penetration site. Free water in the ground substance is still on the increase and at about this time it reaches a maximum which is maintained for a considerable period thereafter. The relationship between free water and Evans blue-staining material is shown in semidiagrammatic form in plate II. One interpretation of the results described may be that the material which results from enzymatic activity of the larvae and which binds the blue dye, is removed from the site of its formation by the host circulation soon after being formed. This seems even more probable when we note that there is also a decrease in glycoprotein shown by the Hotchkiss method. An alternative explanation might be that the extracellular materials have returned to their original state of aggregation and no longer bind the Evans blue. There is no direct evidence that this is the case. A third explanation should also be considered. That is, that the phenomenon may center around a transient capillary permeability change. Changes in capillary permeability probably accompany action of enzymes on acellular glycoprotein constituents of the capillaries.[1] In our routine infections, where relatively small numbers of larvae have been used, the capillaries are not markedly changed in appearance and are not hyperemic. Rarely, superficial capillaries in the immediate vicinity of penetrating larvae show changes in their enclosing glycoprotein similar to the early changes in the basement membrane.

Skin penetration by *Strongyloides* is accompanied by changes similar to those discussed above but there is no evidence of glycoprotein secretion. These organisms differ from the schistosomes in not hesitating at the base-

35. Oliver-González, J. and González, L. M. 1949, Release of the $A_2$ isoagglutinogen-like substance of infectious organisms into human blood serum. J. Infect. Dis. **85**: 66–71.

ment membrane to form tunnels parallel to its surface so that they reach the deeper layers of the dermis even more rapidly than do the cercariae. *Ancylostoma* also causes similar changes but the alteration of the ground substance during penetration of this form is even more marked than with the schistosomes.

We believe that in their passage through the skin, the helminths just discussed discharge enzymes that soften and change to a soluble state the acellular barriers to their progress. The reactions in the basement membrane and ground substance, demonstrated by histochemical means, are considered to be analogous to those described by other investigators as depolymerization effects on connective tissue,[1,7] such changes often being associated with collagenase activity.

At this point we should like to emphasize that one of the skin-penetrating forms does not cause changes exactly similar to the types described. Skin penetration by *N. muris* larvae is not accompanied by an extensive alteration of the glycoprotein, and no Evans blue is found around the penetration site. There is, however, a local increase in free water similar to that seen in the other species studied. As will be brought out later, these differences can be correlated with an absence of enzymes of the type possessed by the schistosomes and *Strongyloides* and argue strongly in favor of a different group of enzymes or of different mechanisms of penetration in the case of *Nippostrongylus*.

We have suggested that the basement membrane and ground substance offer a significant barrier to the penetrating helminths. There is considerable evidence in favor of this view. First, the organisms almost immediately penetrate the epidermis but are delayed and change their course when they reach the basement membrane. A perceptible interval is necessary before this is altered sufficiently to allow further progress in the case of the cercariae. Secondly, in old animals and in hypophysectomized animals in which the basement membrane is thickened and the ground substance is more highly polymerized,[1,7,9] resistance to helminth penetration is observed. In the old animals, cercariae are often retained above the basement membrane for considerable periods of time and may be prevented from penetration to the dermis for as long as 24 hours. Also, the production of soluble products in the ground substance is delayed as compared to that in younger animals. In these old animals, the cercariae retained in the skin are always above and resting on the basement membrane. When young hypophysectomized animals are exposed to cercariae and other larvae, many are unable to pass through the membrane to the dermis, while others are retained in the dermis for longer periods of time than in normal animals of the same age, and the production of soluble materials in the ground substance is delayed even longer than in the old animals. These findings should be considered in relation to the nonspecific "age resistance" or "age immunity" to helminths which has been recorded for a variety of forms. The older animals and the hypophysectomized animals resist penetration because their extracellular glycoprotein is in a different state of organization than in young normal hosts. This, in itself, increases resistance to penetration and may play an important role in the increased immunity observed with age. It might be argued that retention of larvae in the skin of older animals not previously exposed to the species in question is due to an increased immunity developed as a result of the greater probability of exposure to related antigens

with increased age. However, this retention of larvae and delay of penetration is also found in young hypophysectomized animals in which no such previous exposure to antigen is possible. This relative "immunity" by virtue of state of organization of the glycoprotein might apply not only to penetration of the skin but also to the penetration of other tissues where basement membranes beneath epithelia must be penetrated or where the ground substance of connective tissue must be traversed.

A fourth item which suggests that glycoprotein membranes offer a considerable barrier to the progress of helminth larvae deserves mention. This is in connection with their activities against membranes not usually penetrated under normal conditions of infection. From a number of experiments not described in the body of this report, we have found that cercariae of the schistosomes and larvae of *Strongyloides* are able to pass the thin basement membrane found beneath the external epithelium of the rabbit cornea. Material stained by the Hotchkiss method shows that the changes which take place with penetration are similar to those seen in penetration of the basement membrane of the skin. However, if cercariae or *Strongyloides* are placed on the mesenchymal epithelium of rabbit cornea, they are unable to pass through the much thicker glycoprotein-containing material found in the membrane of Descmet. With the Hotchkiss method, some local swelling, alteration and superficial pitting of Descmet's membrane can be seen but the larvae are unable to alter it sufficiently to accomplish penetration.

Our histochemical results to date give us little information that can be applied to the problem of host specificity. Slight differences in larval penetration enzymes might conceivably permit them to pass through the basement membrane of one host more readily than through that of another. This type of condition is suggested by a comparison of penetration of the rat skin by *S. ratti* and *S. simiae*. The parasite abnormal to the rat, *S. simiae*, is able to penetrate but seems to do so somewhat more slowly than *S. ratti* and to produce a slightly lesser quantity of soluble glycoprotein, as indicated by the Evans blue response.

As we have previously pointed out, mechanisms similar to those just dis-

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PLATE VI

FIG. 1–4.—Evans blue response to *S. ratti* larvae during penetration of the skin of 6-month-old rats. Frozen-dried, 15 μ sections cleared in immersion oil, ×116.

FIG. 1.—Fifteen-minute penetration: Larva at right (L), just entering the dermis, is surrounded by Evans blue-binding material. Area at upper left has been penetrated by larva which is not visible in this section.

FIG. 2.—Fifteen-minute penetration in cortisone-treated rat. The reaction is more intense than in fig. 1 and it is limited to the epidermis and upper dermis.

FIG. 3.—Ninety minutes after exposure of normal rat to larvae: The skin appears normal, with no accumulations of Evans blue.

FIG. 4.—Fifteen-minute penetration in hypophysectomized animal. The most intense color is found in the deeper portion of the dermis and surrounds the penetrating larva.

FIG. 5.—Ferrocyanide technique: 15-minute penetration by *S. ratti* showing increase in free water immediately around penetration site, ×116.

FIG. 6–8.—Hotchkiss-stained, frozen-dried, 15 μ skin sections comparing the basement membrane (bm) of a normal 16-month-old rat (fig. 6) with that of a normal 6 month-old rat (fig. 8), and a hypophysectomized 6-month-old rat (fig. 7), ×260.

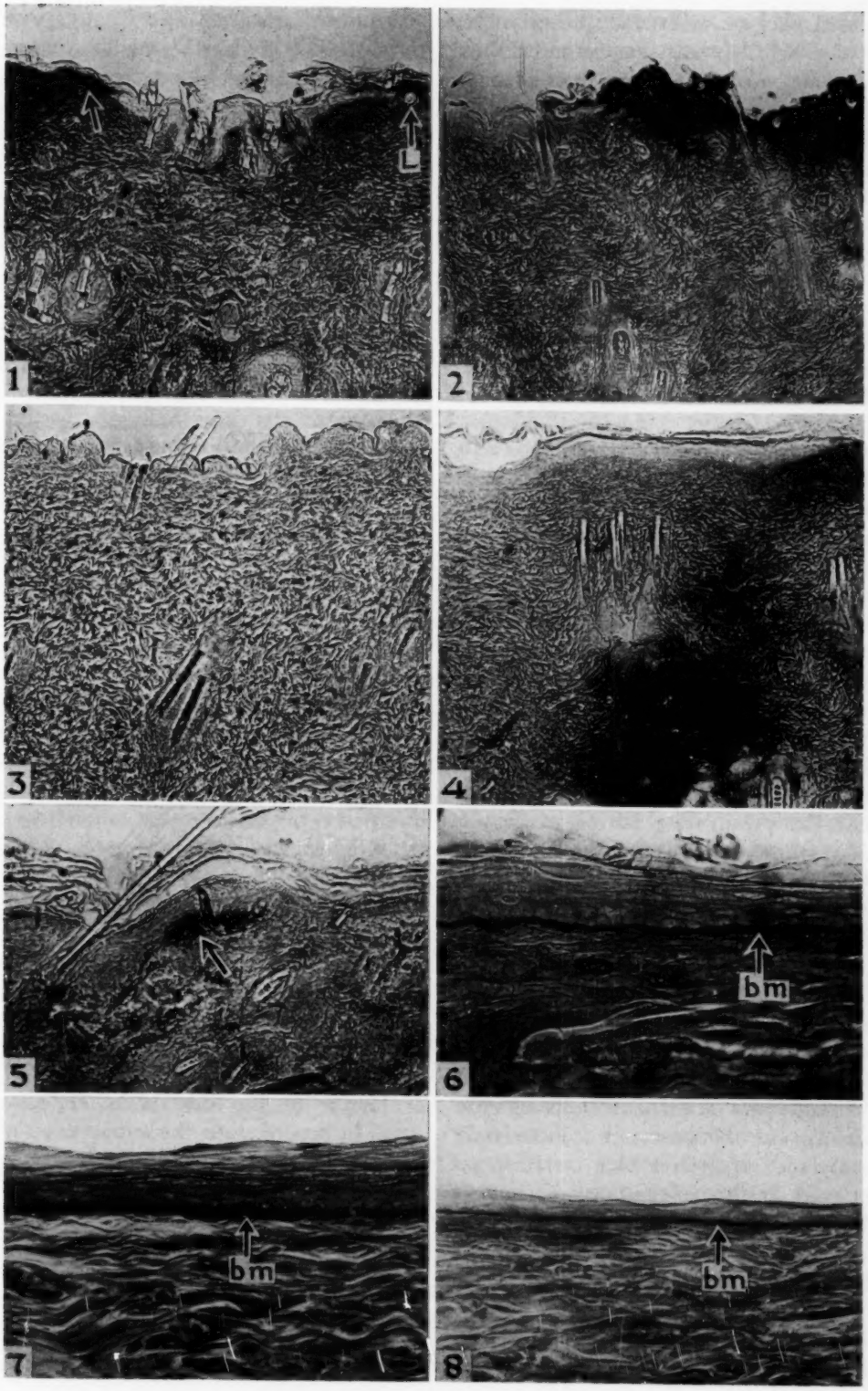


PLATE VI

cussed may be utilized by other stages in the schistosome life cycle in their penetration of hosts or in their passage through tissues. Miracidia cause changes in the glycoprotein of the snail epidermis as they penetrate and possess glands that resemble those of the cercariae in their staining characteristics and in their content of granular carbohydrate-containing protein. The miracidia within viable eggs in host tissues also secrete a Hotchkiss-positive material which is apparently identical to that produced by the free miracidia and the cercariae. We have observed that the changes which take place in the host glycoprotein close to these viable eggs in the tissues are not found around infertile or nonviable eggs. One is tempted to associate these changes and the ability of the egg to pass through the tissues with the secretion of the miracidium within the egg. However, the alterations may well be due to the host response to the presence of a foreign protein and may not be changes imposed on the host by parasite secretions.

We have seen no clear cut evidence that the penetrating larvae of *Trichinella* cause changes in the basement membranes of the intestinal epithelium. It has been suggested by other investigators that both mechanical penetration, aided by a stylet, and proteolytic enzymes are involved.[25] The penetration of striated muscle fibers by the next generation of larvae is associated with various changes, some of which might be related to larval secretions used in penetration. However, we have only rarely seen an Evans blue response associated with penetration of muscle fibers. After the muscle is invaded, there are extensive changes which result in the isolation of the larvae in a capsule which stains intensely with the Hotchkiss method. As in areas of formation of connective tissue or of protease action on connective tissue, the area of capsule formation is strikingly metachromatic following toluidine blue staining.[36] Calcification starts in these capsules, and in those just beginning to show calcium granules there is a decrease in the intensity of Hotchkiss staining that can be correlated with calcification. This decrease may be similar to that recorded in other sites of calcification,[37] where the change is correlated with a change in degree of polymerization rather than with removal of material having active groups.

As might be expected, specific immunity to an organism decreases the rate of penetration as shown by the Evans blue response. This was demonstrated only in the *Strongyloides* since no other species were used in these experiments. It is known from in vitro tests that immune serum will act against the oral, anal and excretory pore exudates of young worms to cause formation of precipitates. There is also evidence that the secretions of the worm will act as antigens to produce the reaction.[18] As we have shown, the secretions of some of the larvae possess collagenase activity and it is probable that some of the immunity is "anti-enzyme" in nature.

We have found that in the penetration of *S. ratti* in the normal non-immune animal treated with cortisone, there is a complete suppression of wheal production, there is a delay in the time of appearance of the Evans blue spots and there is a greater retention of larvae in the dermis at 60 minutes. In accord with the suppression of wheal formation, the local increase in

36. Follis, R. H., Jr., 1951, Effect of proteolytic enzymes and fixation on metachromasia of skin collagen. Proc. Soc. Exper. Biol. & Med. **76:** 272–273.

37. Cobb, J. D. 1953, Relation of glycogen, phosphorylase and ground substance to calcification of bone. Arch. Path. **55:** 496–502.

the amount of intercellular water, as shown by the ferrocyanide reaction, is not as great as that normally found within a few minutes after penetration starts. Several speculations can be made concerning these effects. Cortisone's antiinflammatory and antiallergic activities are known, and it is possible that the wheal suppresion is related to these phenomena. If we consider the wheal response to be due to the presence of some simple toxic agent acting on capillary permeability, then the cortisone activity might be directed in this case against changes in the capillaries. It might also be argued that this particular phenomenon of wheal formation is a sensitivity reaction associated with an antibody response. In this case, the cortisone might conceivably act by interfering with circulating antibody, as it is said to do under certain conditions.[38] Another effect of cortisone that might enter into our picture is a direct interference with enzyme activity such as that claimed in the case of cortisone inactivation of hyaluronidase.[39] Again, we do not wish to suggest that the picture is strictly analogous but rather would like to point out the possibility of alternative explanations.

In the cortisone-treated animals, there is a lag in the Evans blue response with helminth penetration. This is probably due to alteration or removal of the less highly polymerized material, which might react first, by the drug treatment. The initial Evans blue response is intense and is more restricted to the immediate area around the parasite because the ground substance is less fluid and is not able to imbibe water as readily. The material stained by Evans blue is, however, removed almost as rapidly from its site of production in the cortisone-treated animal as it is in the untreated animal.

Antihistamines, given intravenously in high concentrations, have essentially no effect on penetration or on the Evans blue response. The work with these substances was included since it has been pointed out that there is a similarity between the effects produced by the intravenous dye technique used here and the ones used in the study of histamine "blueing." The loss of Evans blue response after a period of time might improperly be considered similar to the "immunity" or refractory period produced by intradermal histamine.[28] However, as we have previously stated, the reaction of areas penetrated by larvae is not like those in the histamine experiments and a second response can be elicited in the same area. The antihistamines do have a tendency to suppress the wheal formed when *Strongyloides* larvae penetrate and they also decrease the intensity of the ferrocyanide reaction but they do not affect the production of Evans blue spots about the penetration sites.

The results of the histochemical studies give indirect evidence that the penetration of the skin is associated with enzymatic secretions of larvae. These secretions attack the basement membrane and ground substance, altering them in such a way as to suggest that they are depolymerized. Our investigations of living larvae, larval homogenates and extracts give direct evidence of the presence of enzymes capable of causing the changes observed in the extracellular materials during penetration. It is not possible at the

38. Fishel, E. E. 1952, Hypersensitivity and the hyperadrenal state. Connective tissues, Transactions of the third conference, New York, Josiah Macy, Jr. Foundation, pp. 117–166.

39. Winter, C. A. and Flotaker, L. 1950. Influence of cortisone and related steroids upon the spreading effect of hyaluronidase. Federation Proc. **9**: 137–138.

present time to characterize precisely the nature of the enzyme or enzymes produced by larvae as they penetrate the skin. However, we can make some generalizations as to their nature from the effects they produce and may further point out that there are similarities and differences in the mechanisms of penetration by different species.

The mechanisms of penetration of the skin by the larvae of schistosomes, *Strongyloides* and *Ancylostoma* appears to be similar. In vivo, their secretions act on the basement membrane and ground substance, softening them and producing soluble products so that there is a subsequent decrease in stainable glycoprotein. The in vitro activity of these secretions against frozen-dried skin sections not only affects the basement membrane and other polysaccharide-containing protein but also indicates a more general proteolytic activity. All three groups of organisms are able to digest gelatin but, in our experiments using the Anson method,[31] were unable to attack hemoglobin. The latter observation suggests that the proteolytic activity is not allied to that of trypsin. These organisms are all capable of acting on an azocoll substrate, releasing dye bound to collagen. In their coupling of this ability with the ability to digest gelatin, they closely resemble the collagenase (k-toxin) of *Cl. welchii*.[29,30] Other investigations, to be published at a later date, further demonstrate the larval enzymes' similarity to those of *Clostridium* and their lack of tryptic activity.

It is more difficult to evaluate the effect of larval extracts injected intradermally on the spread of dye. A "spreading effect" can be demonstrated not only in those species having collagenase activity but also in the extracts prepared from *N. muris*, *T. spiralis*, *R. pellio* and plagiorchid cercariae. Some of the most extensive spreading was found following the injection of *N. muris* extracts, although in this species we have been unable to demonstrate either collagenase or hyaluronidase. We have been unable to demonstrate hyaluronidase in any of the extracts of larval nematodes tested and other investigators have been unable to demonstrate this enzyme in schistosome cercariae.[4] This is not in general agreement with the results reported by other workers,[13,15] who have obtained evidence suggestive of the presence of hyaluronidase in *S. mansoni* as a result of viscometric tests and "spreading factor" tests. A recent method described by Lincicome[40] as a streptococcal decapsulation test has been used by Evans[16,17] to demonstrate an enzyme in *S. mansoni* cercariae. This enzyme acts on the mucoid envelopes of the bacteria in a manner similar to that of the hyaluronidase "complex" enzymes.

These apparently divergent results can be reconciled to a certain degree by recognizing that "spreading" can be caused by nonrelated substances not containing hyaluronidase and that enzymes other than hyaluronidase (in the strict sense) can act on mucopolysaccharide or polysaccharide-containing protein.

## SUMMARY

Histochemical studies have been made of host tissues being traversed by infective helminth larvae, with particular emphasis on the condition of the extracellular glycoprotein of the dermis. The larvae studied include those of *Schistosoma mansoni*, *Schistosomatium douthitti*, *Strongyloides simiae*, *Strongyloides ratti*, *Ancylostoma caninum*, *Nip-*

40. Lincicome, D. R. 1953, A streptococcal decapsulation test for detection of hyaluronidase activity in animal parasites. Exper. Parasitol. **2**: 333–347.

*postrongylus muris* and *Trichinella spiralis*. The results were as follows:

1. Profound alterations of the extracellular glycoprotein-containing material of the skin, notably the basement membrane and ground substance, accompany penetration by the schistosomes, *Strongyloides* and *Ancylostoma*. Related changes are noted in tissues containing schistosome eggs and in the snail being penetrated by miracidia.

2. The larval secretions cause a disappearance of the basement membrane at the site of penetration, formation of water-soluble glycoprotein in the dermal ground substance and an increase in free water in the ground substance.

3. Helminth penetration causes less alteration of acellular substance in aged rats than in young rats and is correspondingly inhibited. It is of interest that in the old animals, the basement membrane is thicker and is presumably more highly polymerized, as is the ground substance.

4. In young hypophysectomized animals, there is also a reduction in the degree of glycoprotein alteration with penetration, again correlated with inhibition of penetration. It is considered significant that these animals, like aged rats, have thicker basement membranes and more highly polymerized basement membranes and ground substance.

5. Penetration of the skin is inhibited to various degrees in immune animals and in cortisone-treated animals.

6. Intravenous antihistamines have no effect on the rate of penetration or on the glycoprotein changes observed histochemically.

7. Skin penetration by *N. muris* is not accompanied by the extensive changes in glycoprotein found with the other skin-penetrating forms studied. Its activity does cause an increase in the free water of the ground substance.

8. Penetration of tissues by *T. spiralis* is also unaccompanied by immediate changes in host glycoprotein of the type described for other species. However, progressive changes in the glycoprotein capsule that forms about the larvae in muscle are described.

9. Enzymatic studies of the larvae listed reveal a correlation between the presence of a collagenase-like activity and the ability to cause changes in the glycoprotein of the host. The larvae of *S. mansoni*, *S. douthitti*, *S. simiae*, *S. ratti* and *A. caninum* possess this enzyme or enzyme complex which is capable of releasing dye from azocoll and is also active against a gelatin substrate. Collagenase is not present in larvae of *N. muris*, *T. spiralis* and *Rhabditis pellio* nor in the plagiorchid and strigeid cercariae tested.

10. It is the conclusion of the authors that the mechanism of penetration of those parasites which cause changes in the basement membrane and ground substance of the host skin is related to the production of a collagenase-like enzyme. Alterations of the states of organization of the hosts' glycoprotein are reflected in changes in the ability of the parasites to penetrate.

# PATHOGENESIS OF *LISTERIA MONOCYTOGENES* INFECTIONS IN NATURAL HOSTS

## I. RABBIT STUDIES

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In a review article on listeriosis in man and animals published in 1951 Girard and Murray[1] stated, " . . . investigation is necessary to establish the avenue of infection and the mode or modes of transmission." The development of this knowledge has been delayed by certain difficulties in isolating *Listeria monocytogenes* from tissues and because more investigations are necessary for an understanding of the occurrences in nonfatal listeria infections.

Recorded studies on pathogenesis are noteworthy. Stanley[2] isolated a lipoid fraction from listeria cells which was capable of stimulating a monocytosis in rabbits. Murray, Webb, and Swann[3] fed cultures orally to 6 young rabbits and produced a disease resembling the natural infection in 3. Pirie[4] fed culture to rabbits, mice, and gerbilles and produced a disease process. Gill[5] instilled culture in the stomach of a rabbit which remained apparently unaffected. He placed inoculum into the nasal cavity of a rabbit, which produced a purulent nasal discharge followed by recovery. This investigator also placed broth culture into the rumen of a sheep every day for 7 days, which resulted in a temperature rise. Two out of three sheep developed encephalitis after the culture was instilled in the nasal cavity. Julianelle[6] was unable to induce a disease in rabbits and monkeys by intranasal drip or by throat sprays of broth culture. He did establish generalized infection in mice which were given culture to drink. Graham et al[7] produced encephalitis in 1 of 2 pigs exposed conjunctivally. Gray et al[8] reported a similar result in a rabbit. Jungherr[9] instilled culture into one lamb intranasally and another lamb conjunctivally, after which both animals developed fever and diarrhea, followed by recovery and agglutinin pro-

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1. Girard, K. F. and Murray, E. G. D. 1951, *Listeria monocytogenes* as the cause of disease in man and animals, and its relation to infectious mononucleosis from an etiological and immunological aspect. Am. J. M. Sc. **221**: Ser. **2**: 343–352.
2. Stanley, N. F. 1949, Studies on *Listeria monocytogenes*. I. Isolation of a monocytosis-producing agent (MPA). Australian Exper. Biol. & M. Sc. **27**: 123–131.
3. Murray, E. G. D., Webb, R. A. and Swann, M. B. R. 1926, A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. J. Path. Bact. **29**: 407–439.
4. Pirie, J. H. H. 1927. A new disease of veld rodents, "Tiger river disease." So. Afr. Inst. Med. Res. Pub. **3**: 163–186.
5. Gill, D. A. 1933, Circling disease: A meningoencephalitis of sheep in New Zealand. Notes on a new species of pathogenic organism. Vet. J. **89**: 258–270.
6. Julianelle, L. A. 1940. The function of *Listerella* in infection. Ann. Int. Med. **14**: 608–620.
7. Graham, R., Levine, N. D. and Morrill, C. C. 1943, Listerellosis in domestic animals. Univ. Ill. Bull. 499.
8. Gray, M. L., Stafseth, H. H., Thorp, F., Jr., Sholl, L. B. and Rile, W. F., Jr. 1948, A new technique for isolating listerellae from the bovine brain. J. Bact. **55**: 471–476.
9. Jungherr, E. 1937, Ovine encephalomyelitis associated with *Listerella* infection. J.A.V.M.-A. **91**: 73–87.

duction. Another sheep inoculated by way of the carotid artery developed encephalitis. Olson et al[10] made an important contribution by injecting several sheep parenterally and studying their thermal and blood cell responses. In their investigations a temperature rise accompanied by a neutrophilic leucocytosis generally followed injection. Intracerebral and intracarotid injections resulted in encephalitis, but inoculations by other routes did not affect the central nervous system. In another experiment Olson et al[11] detected a bacteremia in 2 of 7 sheep after *L. monocytogenes* was placed in the rumen. Olafson[12] attempted unsuccessfully to produce encephalitis in sheep by traumatizing the nasal mucosa with a brush, following intranasal instillation of culture. Graham et al[7] were able to produce a fatal encephalitis in 13 out of 14 sheep inoculated intravenously. Intragastric exposures in a sheep and a horse were followed by a temporary illness.

These studies emphasized the production of encephalitis or death to demonstrate that the clinical problem had been reproduced. Relatively little attention was given to the infections which were followed by recovery.

The purpose of the present investigation was to learn more about the reactions following the union of this bacterial parasite with natural hosts representing both the small mammal and ruminant groups. The hosts chosen were rabbits and sheep. The sheep experiments are described in a following paper.[13] In this study it was necessary to make a distinction between clinical disease and infection. Clinical disease was not used as an index of parasitism since it was considered only an overt manifestation of the infectious process. Infection was regarded in the sense that it need not have apparent detrimental effects but that certain host responses might indicate invasion by the bacterial parasite.

The following facts were sought:

1. Portals of invasion traversed by *L. monocytogenes*.—To determine the hosts' response to invasion by *L. monocytogenes*, it was first necessary to study the results of parenteral injections. This was followed by exposure of mucous membranes likely to be contaminated in nature—the nasal mucosa, vagina, digestive tract, and conjunctiva.

2. Modes of bacterial escape from the host.—Epidemiological information was to be gained by discerning these pathways. It was also desired to know if *L. monocytogenes* would persist on the mucous membranes with which it was placed in contact. With these views in mind, the following materials were cultured: nasal mucosa, pharynx, certain segments of the genital tract, contents of the duodenum and colon, urine, and bile.

3. The pathology of listeria infections.—The pathological results of listeriosis were studied to correlate tissue changes both with alterations of function and the escape of the bacterium to the exterior.

4. The distribution of the bacterium within the hosts.—The intention here was to establish infection pathways

10. Olson, C., Jr., Cook, R. H. and Blore, I. C. 1950, The reaction of blood cells in experimental listeriosis of sheep. Am. J. Vet. Res. **11**: 29–40

11. Olson, C., Jr., Bagdonas, V., Rollins, C. L. and Blore, I. C. 1953, The relation of silage to listeriosis in sheep. Am. J. Vet. Res. **14**: 202–208.

12. Olafson, P. 1940, *Listerella* encephalitis (circling disease) of sheep, cattle and goats. Cornell Vet. **30**: 141–150.

13. Osebold, J. W. and Inouye, T. 1954, Pathogenesis of *Listeria moncytogenes* infections in natural hosts. II. Sheep studies. J. Infect. Dis. **94**: 67–78.

when possible and to determine the degree of dissemination of the bacterium within the animals. This involved culturing the following tissues: liver, spleen, kidney, lung, medulla oblongata, olfactory bulbs, blood, bone marrow, and certain lymph nodes.

## MATERIALS AND METHODS

*Animals.*—The rabbits were of the New Zealand White breed. They were young adults, except for the intragastrally exposed group, which was composed of 3-month-old specimens.

*Culture.*—The culture employed in all experimental inoculations and used for antigen in serological tests was obtained from the medulla oblongata of a sheep which had died of listeriosis in the Davis area during February, 1952 (culture 4-52). The organism was typical of the genus as determined by colonial and cellular morphology, staining reaction, carbohydrate reactions, and its capacity to produce keratoconjunctivitis in the eye of the guinea pig as described by Julianelle and Moore.[14] Sheep and rabbit antiserums prepared against this strain (4-52) agglutinated *L. monocytogenes* antigens prepared from 3 strains obtained through the American Type Culture Collection.

Since the culture (4-52) had been held on tryptose agar slants in the refrigerator for 5 months before its first use as experimental inoculum, its pathogenicity was determined and its virulence enhanced by a passage through mice and a subsequent passage through a rabbit. These animals were killed in 48 to 96 hours by the infection. Because the experiments extended over a period of some months, it was the practice to make a fresh rabbit passage with this culture each time a group of animals was to be exposed.

In preparing inoculum the culture was seeded to small volumes of tryptose broth which were incubated at 37 C for 16 hours. They were then examined for purity, pooled, and centrifuged if a denser suspension was desired. Direct counts were made in a Petroff-Hausser bacterial cell-counting chamber to obviate the problem of clumping.

*Methods of studying the infection.*—Several criteria besides encephalitis and mortality were used to determine the effects of the parasitic residence of *L. monocytogenes* in the mammals. Prior to exposure and during the first 2 or 3 weeks following exposure the animals were examined daily for alterations in body temperature, weight, and total and differential white blood cell values. Animals held for longer periods were then examined every 2nd or 3rd day. Symptoms were noted on each examination and tests for *L. monocytogenes* agglutinins were made every 2 or 3 days.

The serological test was conducted as follows: The antigen was grown in flasks containing tryptose broth which were incubated at 37 C for 48 hours. The cells were killed with 0.5% formalin. This antigen was adjusted to a reading of tube number 2 of the MacFarland nephelometer. The cells remained evenly in suspension with no tendency toward autoagglutination. Serums were diluted in doubling serial dilutions, starting with the 1:25 dilution, and were mixed with 1 ml of antigen. The racks containing the serum and cell mixtures were held at 37 C, with a reading made after 24 hours and a final reading at the end of 48 hours.

Euthanasia, when required, was performed in the rabbits by injecting 10 ml of air intravenously. Many tissues of the autopsied animals were subjected to bacteriological examination. These tissues were aseptically removed from the animals, a different set of sterile instruments being used to obtain each tissue. About 1 to 2 g of tissue from each organ were ground with a mortar and pestle without the addition of an abrasive substance. Several portions of the paste-like tissue were then streaked on cow blood agar plates and incubated at 37 C. If repeated examinations failed to reveal significant growth, the plates were discarded after 5 days. When minute colonies appeared which were grayish-white in reflected light and were surrounded by a zone of $\beta$-type hemolysis, their cells were examined by Gram's staining. If the cells were small gram-positive rods of a morphology typical of *Listeria*, an agglutination test was next performed. This was a spot test made on a slide by first mixing a few drops of saline and a mass of the colonies in question to form a suspension. A loopful of rabbit antiserum was then added and mixed. Positive reactions appeared rapidly and were easily read.

In many instances the identity of the isolates was further confirmed by determining their capacity to produce typical turbidity in tryptose broth in the absence of serum and by testing the biochemical reactions in carbohydrate mediums. These additional steps were followed in the rabbit work when the organism was found in sites normally containing a mixed flora, such as the pharynx or intestinal contents.

When some materials such as intestinal con-

14. Julianelle, L. A. and Moore, E. 1942, Pathological changes in *Listerella* infection, particularly of the eye. Am. J. Path. **18**: 813–825.

TABLE 1.—*Criteria of infection in some* L. monocytogenes *exposed rabbits.*

| Exposure | Outcome | Temperature | Weight* | Agglutinins | WBC† | Large lympho-monocytes | |
|---|---|---|---|---|---|---|---|
| | | | | | | Percent | Absolute |
| Normal Rabbit | | 103.0 S.D. 0.6‡ | Gained 0.11 | | 10,398 S.D. 2,160‡ | 5.5 S.D. 3.8‡ | 572 |
| Submucosal Inoculation (1314) | Sacrificed 23 days | 106.4 | Lost 0.15 | 1:1600 | 19,950 | 35 | 6,982 |
| Nasal (1322) | Died in 6 days | 107.0 | Lost 0.7 | | 15,150 | 32 | 4,848 |
| Vaginal (1326) | Sacrificed 27 days | 107.2 | Lost 0.1 | 1:3200 | 17,100 | 20 | 3,420 |
| Gastric (1338) | Sacrificed 5 days | 106 | Lost 0.2 | | 18,150 | 19 | 3,448 |
| Conjunctival (1342) | Sacrificed 42 days | 105 | Gained 0.05 | 1:3200 | 14,900 | 20 | 2,980 |

* Change in kg during first 5 days following exposure.
† White blood cells per cu mm of blood.
‡ Standard deviation.

tents were cultured, it was necessary to plate not only on blood agar but also on blood agar containing 0.05% potassium tellurite. The value of this inhibtory medium for listeria studies was described by Gray et al[15] and found to be quite useful.

The work of Gray et al[8] had indicated that cultures from fresh bovine brain could fail to reveal *L. monocytogenes*, while reculture of the material after a holding period might yield positive samples. Therefore, all the crushed tissues in the mortars were suspended in a few ml of tryptose broth and placed in tubes. These tubes were refrigerated at 4 C for 45 days and again subjected to the same bacteriological examination as has been described for cultures made at the time of autopsy. The importance of this procedure is discussed more fully in the following paper.[13]

## EXPERIMENTAL

*Control group.*—Since analysis of subclinical infectious processes was important in this study, it was necessary to obtain certain physiological data for normal animals in the existing environment. Eight rabbits which served as a control group were housed and handled in a manner identical to the inoculated specimens and were under observation for an average of 59 days. Eighty-eight to 271 variants were used in the calculation of these normal figures (table 1). The figures on large lymphocytes and monocytes represent pooled counts, since the work of Conway[16] indicated that both of these cell types appear in the peripheral blood of rabbits in increased numbers from the stimulation of a listeria infection.

Weight gain is expressed as the mean weight gain for a 5-day period. This period was sufficiently long to demonstrate a weight trend but not long enough to mask a brief disturbance in body weight.

Also, the serums of these controls were examined for *L. monocytogenes* agglutinins. Fifty agglutination tests were run with not less than 4 tests on any individual. The titers were negative throughout, except for one observation of an incomplete agglutination in the 1:25 dilution.

*Intravenous inoculations.*—In this experiment 7 rabbits were given varied numbers of listeria cells by intravenous inoculations into an ear vein. The dosages ranged from 25 thousand to 5 billion bacterial cells per kg of body weight, with one animal receiving each dosage level. The severity of effects on the hosts was directly proportional to

15. Gray, M. L., Stafseth, H. J. and Thorp, F., Jr. 1950. The use of potassium tellurite, sodium azide and acetic acid in a selective medium for the isolation of *Listeria monocytogenes*. J. Bact. **59**: 443–444.

16. Conway, E. A. 1939, Reaction of lymphatic tissue of rabbits to repeated injections of *Bacterium monocytogenes*. J. Infect. Dis. **64**: 217–240.

the quantity of inoculum. Doses of 500 million organisms per kg and higher killed the rabbits in 40 hours or less, with wide dissemination of the bacterium in the tissues and the production of acute septicemic lesions. The inoculum of 50 million bacteria per kg approximated the minimum lethal dose by the intravenous route, since it produced a course of subacute infection associated with protracted weight loss, fever, leucocytosis, monocytosis, anorexia, and depression, and was associated with marked production of agglutinins. This animal was sacrificed in a moribund condition on the 25th day after inoculation. Listeria organisms were found in the pericardial fluid on initial isolation and also in the liver on reculture.

The only evidences of a disease process shown by the animal receiving 5 million cells per kg were a low antibody titer, a temporary decrease in body weight, and some higher-than-normal levels for the combined large lymphocyte-monocyte counts. When sacrificed after 31 days there were no apparent lesions nor were any listeria cultures obtained.

Inoculations of 500 thousand bacteria or less per kg of body weight produced no effect on the remaining 3 animals in the group, as determined by the methods used in this experiment. One exception was the appearance of trace agglutinin reactions in the 1:25 dilution, but for practical purposes it could be stated that a single intravenous dose in this range was below the antigenic threshold for measurable agglutinin production.

None of the animals in the intravenous group developed encephalitis. The brains of 2 animals receiving the higher doses contained listeria cells. However, they succumbed rapidly, and recovery of the organism from the blood at autopsy made this finding of uncertain importance.

Hemocultures were made at 24-hour intervals in the groups injected intravenously and submucosally. These were invariably negative, which made it appear that the clearance of the bacterial cells from the blood was efficient enough to eliminate a bacteremia or at least maintain it at such a low level that the sampling of 0.5 ml was not likely to disclose the presence of blood infection.

*Submucosal inoculations.*—Submucosal inoculation was considered a useful parenteral route because the bacterial cells in the experiments to follow would be located under the mucosa if they were able to invade that barrier successfully. Nine male rabbits were injected submucosally in the prepuce. Dosage ranged from 25 thousand to 5 billion bacterial cells per kg with 3 rabbits receiving the highest dose. All of the specimens developed a local inflammatory reaction at the site of injection. Swelling and induration of the prepuce in direct proportion to the quantity of inoculum suggested the presence of bacterial toxins. The animals receiving the largest dose of cells developed within 24 hours a prepucial swelling the size of a walnut. This area was very painful and bright red in color. The wall of the prepuce necrosed in some instances and released a light yellow, moderately viscid pus.

Of the three animals receiving 5 billion listeria cells per kg, only one died from the infection. After 4 days this animal developed symptoms referable to the central nervous system and died on the 7th day following inoculation. It developed a very high fever, a mild monocytosis, rapid loss of body weight and complete anorexia. Three days before death there were demonstrated the nervous symptoms of stamping with the forefeet, involuntary muscular twitching of the ears, twitching of the shoulders, stupor, and briefly assumed atti-

FIG. 1.—Brain of intranasally exposed rabbit showing pronounced leptomeningitis, with tendency toward perivascular cuffing of directly associated blood vessels in the brain. ×200.

tudes of opisthotonos. Histologically, the brain showed congestion of the meningeal vessels and choroid plexuses, with a mild infiltration of mononuclear cells in the cerebrum. No perivascular cuffs were seen. Listeria cells were found widely disseminated, including such sites as the pharynx, duodenal contents, and urine.

Another specimen receiving this high dosage of 5 billion cells per kg was sacrificed after 21 days, at which time listeria cells were recovered only from the pharyngeal tissue. The 7 remaining animals also developed patterns of reaction which gave evidence of an infectious process (example in table 1). The host response diminished greatly as the size of the inoculum was decreased. All these specimens recovered clinically and were sacrificed 23 to 41 days after inoculation. There were no gross lesions nor was *L. monocytogenes* recovered from these animals. The inflammatory re-

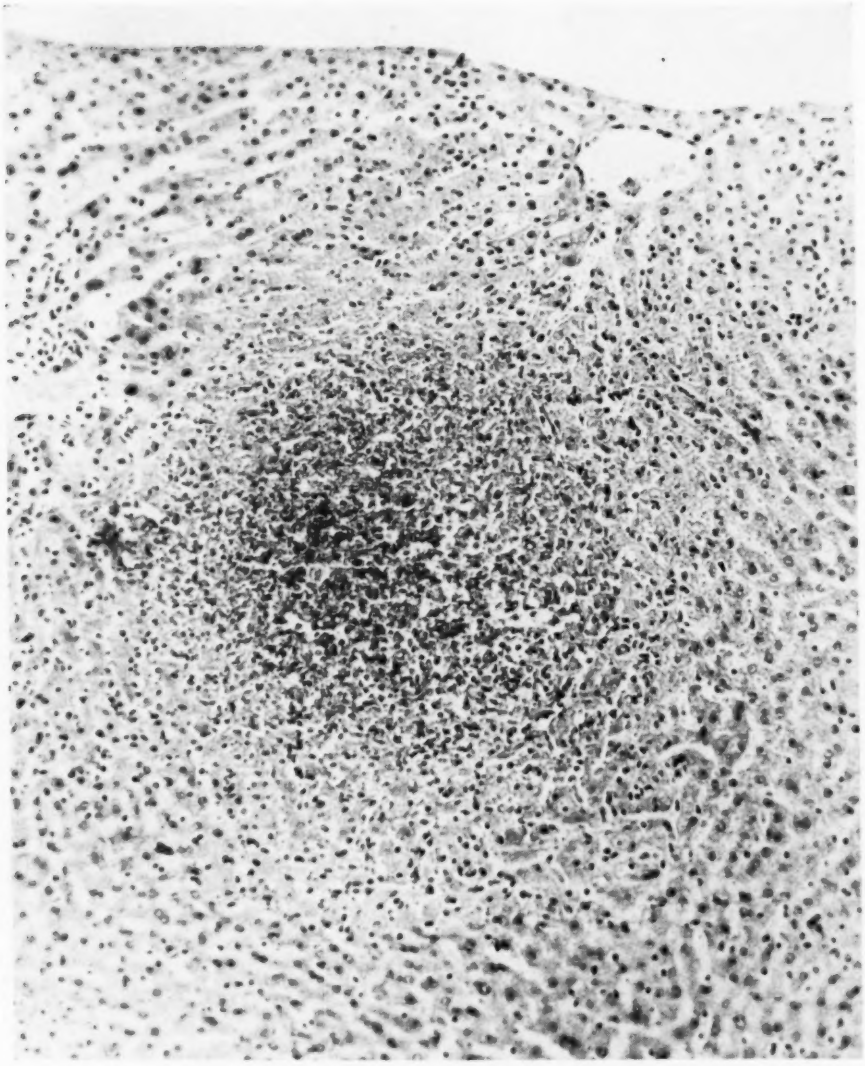

FIG. 2.—Liver of intranasally exposed rabbit showing a focal area of necrosis. ×100.

sponse to the bacterial cells deposited in one location in the tissues seemed rapid and quite effective in preventing an overwhelming septicemic invasion.

In determining the amount of inoculum for the various mucous membrane exposure experiments, the information gained from the submucosally inoculated animals was considered. In this experiment 3 specimens received 5 billion cells per kg of body weight. Two of them survived while the third succumbed. Since this dosage produced all the desired effects of listeriosis in the rabbits but was not overwhelmingly fatal even when placed under the mucous membrane, it was chosen as the dose for membrane exposures.

*Intranasal exposures.*—In the intranasal group the inoculum was concentrated to a volume of about 1 ml. This was instilled as a single dose of 5 billion bacteria per kg. of body weight into the left nostril. The animals were held

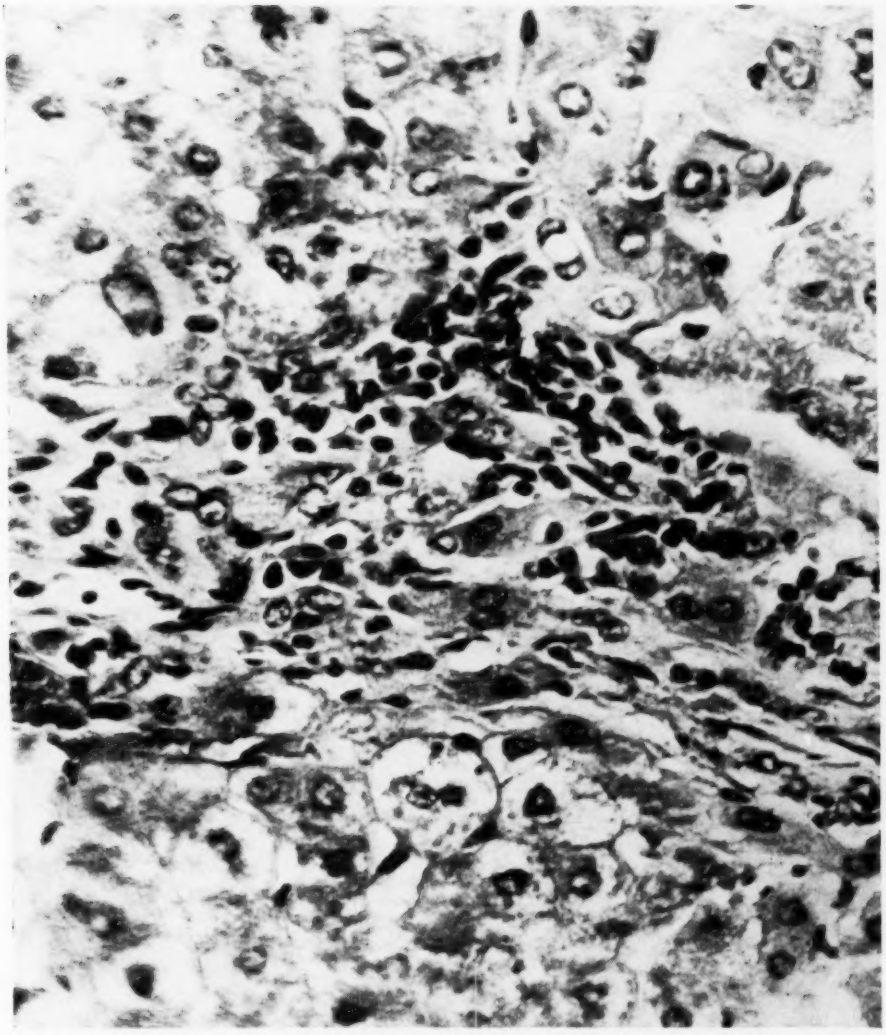

FIG. 3.—Liver (rabbit 1393). Area of mononuclear infiltration in interlobular septae. ×450.

without anesthesia on their backs for a few minutes so the listeria cells might gravitate to the area of the cribriform plate. Undoubtedly some of the inoculum gained entrance to the pharynx and thus to the gastrointestinal tract. Therefore, this was not specifically an intranasal exposure alone but rather an exposure in which it was assured that the olfactory area was included.

There were 7 rabbits in the intranasal group. The animals followed almost identical patterns through the early days of the infection, but some divergences became apparent in the final outcome (example in table 1). The animals experienced a precipitous weight loss, hyperthermia, and an increase in large lymphocytes and monocytes. Twenty-four hours after the exposure they were markedly depressed and were discharging a yellowish-white pus from the nares. After 48 hours the discharge was more copious and was associated with dyspnea, sneezing, and some suppurative discharge from the inner canthi of

FIG. 4.—Cross section of the uterus of an intravaginally exposed rabbit. The dark zone under the myometrium consists of leucocytes and cellular debris. The large amorphous mass in the center is necrosed embryonic tissue. ×30.

the eyes. One animal died in this stage.

Seventy-two hours after exposure disorders of the central nervous system began to appear. This fatal effect was seen in 4 specimens. The symptoms of encephalitis were hyperexcitability, quivering, stamping with the forefeet, and sitting up much like an alert dog with the head held high. Such an action might next be followed by placing the head on the cage floor and rotating it with one side of the face turned up. Of the two animals which showed this head rotation, one faced to the left, the other to the right. If the head was forced to the other side the rabbit would quickly return it to its previous position.

The two which survived reached the bottom of their weight curves on the 9th day and had regained their pre-exposure weights three weeks after infection. Also on the 9th day exudation from the mu-

FIG. 5.—Duodenum of intragastrally exposed rabbit (1338) showing excessive mononuclear leucocytic infiltration in lamina propria. ×200.

cous membranes disappeared.

Many tissues yielded positive cultures only after they had been recultured (example in table 2). The nasal mucosae of the 5 animals that died from the infection contained the bacterium on autopsy. Histologically the area of the cribriform plate had become a disorganized mass of multiple abscesses and diffuse neutrophilic infiltration. The meninges of these 5 animals showed varying degrees of pathological changes. Severe leptomeningitis was seen in some specimens in which there were thrombosis of the meningeal vessels, hemorrhages, and heavy cellular infiltrations with neutrophils and mononuclear cells. Slight cuffing was seen in the Virchow-Robin spaces directly associated with the meningeal inflammation (fig. 1). As would be expected, both the olfactory bulb and the brain stem cultures from these animals contained listeria organisms. The location of the pathological

TABLE 2.—*Some isolations of* L. monocytogenes *following mucous membrane exposures in rabbits.*

| Tissue | Rabbit numbers and exposure routes | | | | | |
|---|---|---|---|---|---|---|
| | Nasal | | Vaginal | | Gastric | |
| | 1322 | | 1325 | | 1338 | |
| | Or | Re | Or | Re | Or | Re |
| Nasal mucosa | + | + | − | − | − | − |
| Pharynx | − | + | − | + | + | + |
| Duodenal contents | − | + | − | + | − | + |
| Colon contents | ... | ... | ... | ... | − | + |
| Urine | ... | ... | − | + | − | − |
| Bile | ... | ... | ... | ... | − | − |
| Iliac node | ... | ... | + | + | − | + |
| Mesenteric node | + | + | ... | ... | − | + |
| Testicle | − | + | ... | ... | − | − |
| Cervix | ... | ... | + | + | ... | ... |
| Uterus | ... | ... | + | + | ... | ... |
| Liver | + | + | − | + | − | + |
| Spleen | + | + | − | + | − | + |
| Kidney | − | + | − | − | − | + |
| Medulla oblongata | − | + | − | − | − | − |
| Olfactory bulb | + | + | − | − | − | − |
| Blood | − | ... | − | − | − | − |
| Bone marrow | + | + | − | − | − | − |
| Lung | − | + | − | + | + | + |

Or, original culture. Re, reculture. +, *L. monocytogenes* recovered. −, negative result. . . . , not cultured.

alterations in the cranial cavities of these rabbits made it appear that the bacterium had grown by extension through the cribriform plate and then ramified over the meninges. While this did result in a central nervous system disorder, it was not analogous to the natural disease as seen in ruminants. The bacterium had also become widely disseminated in other organs resulting in hepatic necrosis (fig. 2) and pulmonary edema and hyperemia. The presence of the organism in the urine of one animal suggested the existence of infective foci in the kidneys. The contents of the duodenum contained the bacterium in 3 of the 5 fatal cases.

Perhaps the most interesting animal from an epizootiological point of view was rabbit 1393, since listeria cells were found in the liver and small intestine of that specimen. From a clinical standpoint the animal had recovered from the infection, yet it was shedding listeria cells 23 days after exposure. The liver of this specimen had rather extensive nests of lymphocytes and monocytes in the interlobular septae, which may have been areas of persistent focal infection (fig. 3). The other rabbit which survived the acute infection had apparently eradicated the parasitic cells from its tissues by the time of autopsy 22 days after exposure.

*Intravaginal exposures.*—The possibility of venereal transmission of listeriosis among rabbits was considered. Experiments were conducted to learn if *L. monocytogenes* could become a resident of the genital mucosa of the doe rabbit, to determine the effects of the bacterium on embryos, and to investigate the possibility for generalized infection of the dam via this route.

The dose of 5 billion listeria cells per kg of body weight was concentrated to a volume of about 0.6 ml. This inoculum was injected through a cannula inserted so that its tip contacted the anterior end of the vagina. The bacteria were allowed to come in contact with the cervices of each specimen by holding the subject with its head lowered for a few minutes.

Seven rabbits were exposed on their 7th day of gestation, at which time nidation of the embryos occurs. The intention was to avoid killing the embryos during their first period of development so that the fetal placenta might expand to contact greater areas of the endometrium, thereby bringing the listeria cells into intimate contact with both maternal and fetal tissue.

The genital tract exposed in this manner proved to be another tissue which could not prevent a listeria invasion. All the specimens responded quite consistently (example in table 1). The immediate invasiveness of the cells and probably their toxic nature were shown by the constant temperature elevations 24 hours after exposure. Agglutinins began to appear between the 6th and 8th days of the infections in all of the specimens. Twenty-four hours after exposure the number of white

blood cells had decreased by about 2000 and then began to increase.

While all these responses to a bacterial parasitism were occurring, there was surprisingly little clinical evidence of disease. Except for small amounts of vaginal discharge from 3 animals, casual observation revealed nothing else suggesting an infectious process. None of the animals died of the infection, but they were sacrificed at varying periods to determine the status of the parasitism at that time.

Autopsy revealed that 5 of the 7 animals had been pregnant with the attachment of embryos and subsequent embryonic death. Incision into an involved uterus revealed a hyperemic wall about 4 mm thick, with the embryos appearing as white caseated masses 5 to 6 mm in diameter. Histological examinations demonstrated suppurative metritis, with great accumulations of giant cells and other leucocytic infiltrations as host reactions to both the pathogenic bacteria and the embryos, which then constituted masses of foreign body debris (fig. 4). It is noteworthy that this process was still going on in animals that were killed as long as 7 days after a normal gestation would have ended. Death of the embryos could not have been an immediate effect of the infection but rather a process that occurred some days after the embryos and listeria cells had shared their role in invasion of the uterine wall.

Animal 1325 also had an acute suppurative cystitis. The fundus of the organ contained a cheesy pus from which listeria cells were recovered (table 2). The iliac lymph nodes, which filter lymph from the genital tract, were enlarged in some of the animals, suggesting that the bacterium had first entered the lymphatic pathways. One rabbit showed the unusual lesion of yellowish amorphous granules around the chordae tendineae in the left ventricle of the heart. Listeria organisms were recovered from these masses.

Another animal was interesting from an epizootiological aspect because it seemed clinically normal 27 days after infection but was shedding listeria cells from the genital tract and in the urine. The kidneys contained leucocytic nests in the cortex and corticomedullary junction, suggesting that the bacteria in the urine were there as a result of generalized infection with foci in the kidneys.

Five more rabbits were exposed intravaginally at varying periods of gestation. They all showed evidences of having been infected. However, death occured in only one specimen, which resulted from a bacterial invasion of its central nervous system.

*Intragastric exposures.*—The gastrointestinal tracts of 7 three-month-old rabbits were exposed to *L. monocytogenes* at a dosage of 5 billion bacterial cells per kg of body weight. The animals were anesthetized with ether, and the dose was instilled by tube into the stomach. Undoubtedly some contamination of the pharnyx and oral cavity occurred as the tube was withdrawn.

While the host reactions were somewhat less pronounced than in previously described groups (example in table 1), the criteria of infection studied indicated that infection probably occurred throughout the group. Antibody titers ranged from 1:400 to 1:6400, except in the instance of one animal which was sacrificed at 5 days. On the basis of physical examination alone these animals were entirely asymptomatic.

Rabbit 1338 was sacrificed 5 days after exposure, and at that time the small intestine appeared somewhat hyperemic. The spleen was enlarged to about 3 times normal size, the liver contained what appeared to be intralobular areas of necrosis, and the lungs

were hyperemic, with some areas of consolidation. The mesenteric lymph nodes were enlarged and somewhat darker than normal. Reculture of the tissues from this specimen (table 2) revealed that the listeria cells were widely disseminated in the host. This animal had apparently demonstrated that listeriosis would occur in rabbits if the cells entered the body by way of the alimentary tract. It was not possible to determine which level of the gastrointestinal tract had experienced the invasion, although the lamina propria of the duodenum was seen to be excessively infiltrated with mononuclear leucocytes, which suggested entry of the bacterium in that area (fig. 5).

Another specimen was sacrificed on the 10th day following exposure. The spleen was enlarged to twice normal size and was the only gross lesion noted. Reculture of the tissues of this animal revealed listeria cells in the pharyngeal tissue only.

The remaining specimens in the group showed only scant pathological alterations, except for varying degrees of splenic enlargement. The tissues of these 5 animals were bacteriologically negative when autopsies were performed 17 to 41 days after exposure.

*Conjunctival exposures.*—Two rabbits were exposed conjunctivally by placing 4 drops of broth culture containing 88 billion cells per ml on the conjunctiva of the left eye.

The host reactions to this procedure were analogous to those observed in other exposure methods (example in table 1). Within 24 hours after the instillation an acute suppurative conjunctivitis had developed which was associated with depression of the host. The lids were swollen and glued shut by the accumulated pus on the second day. Corneal opacity was then becoming apparent. Keratitis became very pronounced but began to clear in a few days in the eye of one animal and the tissue appeared normal 26 days after the exposure. The keratitis was more severe in the other specimen since ulceration developed on the cornea, and opacity associated with repair was still apparent in this specimen when the animals were destroyed at the end of 42 days. Neither animal yielded listeria cultures when autopsied.

The rather unique reaction of *L. monocytogenes* on the mucous membrane of the eye appears to be a graphic demonstration of the bacterium's ability to attack mucous membranes. It was more dramatic in this instance than in other mucous membrane exposures because the reaction was so easily observed.

## DISCUSSION

The rabbits demonstrated their susceptibility to invasion by *L. monocytogenes* through any of the exposure routes employed when the inoculum was of the quantity and virulence used in this study. This makes it impossible to state which portal of entry is likely to be used most often in nature. However, the accessibility of the alimentary tract could make ingestion of the organism a common mode of transmission.

The pathological and bacteriological evidence indicated that the lymphatic pathways from the primary focus of infection were important as an avenue for wider dissemination of the organism. Bacterial invasions were followed by varying degrees of protective efficiency from the host responses so that the complete panorama of end results from parasitism became manifest. The outcome varied from complete recovery to latent infection and acutely fatal disease processes.

Involvement of tissues such as the meninges and brain could very readily effect a fatal outcome. A total of 45

rabbits was either injected with the organism or infected by bacterial invasion through the mucous membranes. Of this total, 9 (20%) harbored the bacterium in the tissues of the cranial cavity. Three of these nine animals died before symptoms referable to the central nervous system had developed. The remaining group of 6 animals that had shown clinical disturbances of the central nervous system was comprised of 1 rabbit exposed intravaginally, 1 inoculated submucosally and 4 exposed intranasally.

There was some indication in brains containing *L. monocytogenes* that the clinical symptoms were out of proportion with the observable tissue changes. This gives rise to the question whether much of the effect on the central nervous system might not be due to a toxic component of the bacterial cell which drastically effects the host even though there may be relatively few organisms in the cranial cavity. Further support for a bacterial toxin comes from the long recognized lesion of hepatic necrosis and from the immediate and pronounced local inflammatory area at the site of submucosal inoculation, which was usually associated with necrosis.

A total of 616 tissues from the rabbits was cultured. Since it was necessary to reculture, twice that number of cultures were made in the rabbit experiments. Of the total tissues examined, 148 were positive for *L. monocytogenes*. These positive specimens were obtained from 19 different animals. On the original examination only 79 cultures were positive. Therefore, 69 tissues containing *L. monocytogenes* became apparent only on reculture. A great diversity of rabbit tissues showed this delayed culture effect; the significance of this finding will be discussed more fully in a following paper.[13]

It is reasonable to anticipate the eventual disclosure of reservoir hosts which might serve to maintain *L. monocytogenes* in such a balance as to render an area enzootic for listeriosis insofar as domestic animals are concerned. Reservoir status usually requires a relatively well-balanced state of parasitism which permits prolonged survival of the parasite and an opportunity for it to escape and gain a new host. Animals with asymptomatic infections, from which the disease agent may escape at several body surfaces, can act as reservoirs. There was an indication in these experiments that rabbits could function in that part of the infection chain. Table 3 lists the rabbit tissues examined from which listeria cells are likely to escape and contaminate the surrounding environment. The liver is included here because of the frequency with which this organ is involved in listeriosis in the lapine host. The opportunity for infected livers to shed *L. monocytogenes* down the bile duct would seem to make this organ important from an epizootiological standpoint. Table 3 groups the animals which experienced acute infection and death within 6 days separately from those which had subacute or chronic infections. The latter group had been eating for varying numbers of days following exposure. This fact would indicate that they would be actively

TABLE 3.—*Isolations of* L. monocytogenes *from rabbits of epidemiological importance.*

| Tissue | Acute Infections* | | Subacute Infections† | | Combined | |
|---|---|---|---|---|---|---|
| | Number positive | Percent total rabbits | Number positive | Percent total rabbits | Number positive | Percent total rabbits |
| Nasal mucosa | 8 | 18 | | | 8 | 18 |
| Pharynx | 9 | 20 | 6 | 13 | 15 | 33 |
| Liver | 9 | 20 | 5 | 11 | 14 | 31 |
| Intestinal contents | 6 | 13 | 3 | 7 | 9 | 20 |
| Urine | 3 | 7 | 2 | 4 | 5 | 11 |
| Genital tract | 2 | 4 | 3 | 7 | 5 | 11 |

* Died in 6 days or less.
† All animals sacrificed by euthanasia.

foraging for food in the wild state. Contamination of the feed and water of wild mammals and domestic livestock could occur from the feces, urine, saliva, and genital excretions of such specimens.

## SUMMARY

1. Instillation of *Listeria monocytogenes* on the nasal mucosa, vagina, conjunctiva or gastrointestinal tract of rabbits resulted in listeriosis. The outcome of the diseases produced ranged from acute fatal infections to complete recoveries, with a percentage of animals persisting as latently infected shedders.

2. Nine rabbits (20%) experienced infection of the structures of the cranial cavity.

3. Evidence was presented suggesting that rabbits might serve as reservoirs for this infection. From 45 infected rabbits *L. monocytogenes* was found in the nasal mucosae of 8 (18%), the pharynges of 15 (33%), the livers of 14 (31%), the intestinal contents of 9 (20%), the urine of 5 (11%), and the genital tracts of 5 (11%).

4. A great variety of tissues failed to reveal listeria cells on original culturing. Of 148 positive tissues, 69 (47%) were positive only after reculture following a holding period.

# PATHOGENESIS OF *LISTERIA MONOCYTOGENES* INFECTIONS IN NATURAL HOSTS

## II. SHEEP STUDIES

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In a previous paper[1] the purposes of this study were more fully outlined. This phase of the investigation was devoted to the listeriosis problem in the ovine host. Sheep were used because they are more commonly affected with *Listeria monocytogenes* than any of the other large domestic animals. Furthermore, since sheep are an important food source, they may have a function in the epidemiology of listeriosis in man.

Gross lesions are generally absent in clinical cases of the disease in the large domestic animals,[2–4] wherein the usual syndrome is that of encephalitis or meningoencephalitis. With few exceptions, *L. monocytogenes* has been recovered only from the brains of ruminants affected with listeria encephalitis.

### MATERIALS AND METHODS

The materials and methods used in the entire study were presented in the previous paper[1] and will not be repeated in detail here.

The experimental sheep were obtained from two sources. Three Rambouillet ewes and the suckling lamb of one of these ewes were raised on the University experimental farm. Twelve 4-month-old lambs were purchased at public auction and were used in the experiment when 11 months of age. They were mixed breeds representing Suffolk, Rambouillet, and crosses of these two breeds.

Euthanasia in the sheep was performed by exsanguination after the animals had been stunned by electrical shock.

The tissues of these specimens were examined bacteriologically as in the investigations on rabbits, except that all the described criteria for identification were used on each isolation from the sheep, to avoid any possibility of confusion with other genera such as the *Corynebacteria*.

### EXPERIMENTAL

*Control group.*—The 16 sheep were subjected to several examinations during a 2-month period prior to use in the exposure groups, and the information thus gathered constitutes the normal data.

Since the histories of contacts by these sheep with *L. monocytogenes* were not known, it was necessary to examine surface tissues for the presence of the bacterium and to determine the levels of agglutinating antibodies. Swabs were taken from the nasal mucosa, rectum, and genital mucosa. The swabs were then smeared over the surfaces of blood agar plates containing 0.05% potassium tellurite. Examinations of the growths on these plates did not reveal *L. monocytogenes*.

A total of 68 agglutination tests were run on these animals, with not less than 2 nor more than 6 determinations being made on any individual. Traces of agglutination were found in low dilution in the serums of several of the animals, but when complete agglutination in the

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2. Graham, R., Levine, N. D. and Morrill, C. C. 1943, Listerellosis in domestic animals. Univ. Ill. Bull. 499.
3. Biester, H. E. and Schwarte, L. H. 1940, *Listerella* infection in swine. J. A. V. M. A. **96**: 339–342.
4. Olafson, P. 1940, *Listerella* encephalitis (circling disease) of sheep, cattle and goats. Cornell Vet. **30**: 141–150.

TABLE 1.—*Criteria of infection in some* L. monocytogenes *exposed sheep.*

| Exposure | Outcome | Temperature | Agglutinins | WBC* | Neutrophils | |
|---|---|---|---|---|---|---|
| | | | | | Percent | Absolute |
| Normal sheep | | 103.3<br>S.D. 0.7† | | 7,740‡<br>S.D. 2,160† | 28.5‡ | 2,206 |
| Submucosal inoculation (637) | Sacrificed 15 days | 105.8 | 1:1600 | 19,250 | 63 | 12,126 |
| Nasal (569) | Not sacrificed | 105.7 | 1:6400 | 17,500 | 74 | 12,950 |
| Vaginal (596) | Sacrificed 16 days | 105.1 | 1:800 | 8,350 | 49 | 4,091 |
| Gastric (598) | Not sacrificed | 105.4 | 1:800 | 16,700 | 76 | 12,692 |

* White blood cells per cu mm of blood.
† Standard deviation.
‡ Todd et al.[5]

1:25 dilution was used as a base reaction, only 3 animals showed titers. Two animals had titers of 1:25, while another specimen reacted in the 1:100 dilution. These results were considered in evaluating the outcome of the exposure groups.

*Submucosal inoculations.*—Three 11-month-old animals were injected respectively with 50 million, 500 million, and 5 billion bacterial cells per kg of body weight. In 24 hours the temperatures had all risen 2 to 3 degrees above the normal mean, and a leucocytosis which was more specifically a neutrophilia had developed. An area of induration appeared at the site of inoculation which was readily apparent but less pronounced relatively than those occurring from rabbit injections. During the first 3 days after the injections there was some evidence of depression and loss of appetite, which was followed then by a return to normal feeding habits. Within 2 weeks all of the animals produced agglutinating antibodies, ranging in titer from 1:400 to 1:6400 (example in table 1).

The local inflammatory lesion subsided in about 5 days in 2 of the sheep, but persisted until euthanasia was performed on the 15th day in the 3rd specimen. The only positive culture obtained from these animals came from the injection site on this sheep (table 2). The other 2 specimens were sacrificed on the 16th and 18th postinjection days.

The transitory lymphopenia following the injection of *L. monocytogenes* into sheep, reported by Olson et al,[6] was not noted in these animals. However, the inoculations did substantiate their results that a circulating monocytosis is not a typical response of the ovine host to *L. monocytogenes*.

From the data obtained in this submucosally inoculated group, it was concluded that experimental listeriosis in sheep could be detected with reasonable accuracy in specimens which harbored the parasite but did not show the dramatic symptoms of a listeria encephalitis.

*Intranasal exposures.*—The group exposed by intranasal instillation consisted of three 11-month-old ewe lambs and one 4-year-old ewe. The inoculum (5 billion listeria cells per kg of body weight) was concentrated to a volume of about 1.5 to 2.5 ml. The instillations were made by permitting the fluid to run backward in the nasal cavity while the sheep were held on their backs with the muzzle slightly raised. The purpose was to assure the contact of the structures in the posterior nasal cavity with

5. Todd, A. C., Wyant, Z. N., Stone, W. M. and Elam, G. W. 1952, On the blood picture of healthy Southdown and Hampshire ewes. Am. J. Vet. Res. **13**: 74–76.

6. Olson, C., Jr., Cook, R. H. and Blore I. C. 1950, The reaction of blood cells in experimental listeriosis of sheep. Am. J. Vet. Res. **11**: 29–40.

TABLE 2.—*Isolations of* L. monocytogenes *from experimental sheep.*

| Tissue | Sheep numbers and exposure routes | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Submucosal | | Nasal | | | | Vaginal | | Gastric | |
| | 637 | | 615–7 | | 1098 | | 591 | | 325 | |
| | Or | Re | Or | Re | Or | Re | Or | Re | Or | Re |
| Nasal mucosa | − | − | − | + | − | − | − | − | − | + |
| Pharynx | − | − | − | + | − | − | − | − | − | + |
| Duodenal contents | − | − | − | − | − | − | − | − | − | − |
| Colon contents | − | − | − | − | − | − | − | − | − | − |
| Urine | − | − | − | − | − | − | − | − | − | − |
| Bile | − | − | − | − | − | − | − | − | − | − |
| Mesenteric node | ... | ... | − | + | − | − | − | − | − | + |
| Iliac node | ... | ... | − | − | − | − | − | − | − | − |
| Suprapharyngeal node | ... | ... | − | + | − | − | − | − | − | + |
| Prefemoral node | − | − | ... | ... | ... | ... | − | − | ... | ... |
| Testicle | − | − | ... | ... | ... | ... | ... | ... | − | + |
| Prepuce | − | + | ... | ... | ... | ... | ... | ... | ... | ... |
| Vagina | ... | ... | ... | ... | ... | ... | − | + | ... | ... |
| Cervix | ... | ... | ... | ... | ... | ... | − | + | ... | ... |
| Uterus (right) | ... | ... | − | − | − | − | − | − | ... | ... |
| Uterus (left) | ... | ... | ... | ... | ... | ... | − | − | ... | ... |
| Mammary gland | ... | ... | ... | ... | − | − | ... | ... | ... | ... |
| Liver | − | − | − | + | − | − | − | − | − | + |
| Spleen | − | − | − | + | − | − | − | − | − | + |
| Kidney | − | − | − | − | − | − | − | − | − | − |
| Rete mirabile | ... | ... | − | − | − | − | − | − | − | − |
| Medulla oblongata | − | − | − | − | − | + | − | − | − | − |
| Olfactory bulb | − | − | − | + | − | − | − | − | − | − |
| Blood | − | − | − | − | − | − | − | − | − | − |
| Bone marrow | − | − | − | − | − | − | − | − | − | − |
| Lung | ... | ... | + | + | − | − | − | − | − | − |

Or, original cultre, Re, reculture. +, *L. monocytogenes* recovered. −, negative result. . . . , not cultured.

the bacterial cells. When, after a few minutes, the animals were released, there was undoubtedly further distribution of the infective liquid to the pharynx and thus to the rumen.

The sheep responded with surprising uniformity to the infection. Reactions analogous to those following injection occurred, except that inappetence and dyspnea were more marked and were associated with a copious nasal discharge (example in table 1). There was some loss of body weight.

All antibody titers exceeded the 1:800 dilution, except in the instance of one specimen (615-7) which was sacrificed after 6 days. In this animal the nasal cavity no longer showed an abundance of suppurative exudate but somewhat more serous fluid and tissue edema than would be considered normal. The suprapharyngeal and mesenteric lymph nodes seemed enlarged and excessively moist. The heart showed a few petechiations along the coronary groove and under the endocardium of the left ventricle. There was possibly an increase in the volume of serous fluid in the serosal cavities. Grossly, the brain appeared normal. In summary it may be stated that there were no striking postmortem lesions, which is the usual finding in listeriosis of ruminants. However, reculture of the tissue demonstrated listeria cells in the liver, spleen, lungs, mesenteric and suprapharyngeal lymph nodes, pharynx, nasal mucosa, and left olfactory bulb (table 2). Histopathological examination of the olfactory tract and medulla oblongata revealed no lesions. The left olfactory bulb contained listeria cells, and it was entirely possible that the organisms had not advanced further than that structure at the time the animal was sacrificed. In all probability the bacterial cells were present in the olfactory bulb as a result of extension through the cribriform plate. A delay of 1 or 2 days in sacrificing this sheep might have permitted an encephalitis to become manifest.

The mature ewe, sheep 1098, was lethargic by nature, but 2 days before being sacrificed there had developed a

very apparent attitude of stupidity and irresponsiveness. The presence of a 1:25 agglutinating titer in this animal prior to exposure gave rise to the question of the specificity of those antibodies for *L. monocytogenes*. It could have been expected that if the antibodies were present from a previous encounter of the host with listeria antigens, an anamnestic reaction or perhaps an immune status would be demonstrated. This did not seem to be the case since her response patterns were not markedly different from those of the other specimens, except for a rather low total leucocyte reaction. The great increase in agglutinin titer did not occur any sooner than in the other animals. The specimen was killed 15 days after the instillation. Autopsy revealed no gross lesions. The interesting finding in this animal was the recovery of listeria cells from the medulla oblongata on reculture of the tissues (table 2). It appeared that this animal was in an early phase of listeria encephalitis when it was sacrificed. The medulla oblongata demonstrated some perivascular cuffs composed of mononuclear cells, and there was pronounced edema in the Virchow-Robin spaces. The tissue was hyperemic but no nests of leucocytes were seen in the parenchyma (fig. 1). The olfactory tract appeared normal.

The remaining specimens were held for further study of their antibody curves. Serum antibodies persisted at a high level since tests 79 days after exposure gave readings of 1:800 and 1:3200.

*Intravaginal exposures.*—Four nonpregnant sheep were exposed intravaginally to determine whether the genital mucosa might serve as a favorable environment for listeria organisms and to ascertain the capacity of the gential tract to prevent invasion of the bacterium by this route.

The listeria organisms were instilled intravaginally at the rate of 5 billion cells per kg of body weight by injection through a catheter so that the bacteria were deposited deep in the vagina in the region of the cervix. The sheep thus exposed were confined in such a manner that the vagina was essentially vertical. The specimens were freed after it was felt that the volume of approximately 1.5 ml of fluid had come in contact with the cervix.

The group consisted of three 11-month-old ewe lambs and one mature ewe. Within 2 days all 4 animals had developed noticeable vulvar edema and hyperemia of the vulvar mucosa. This was followed by the appearance of scanty amounts of yellowish-white pus at the ventral commissure of the vulva. These evidences of vaginitis had diminished or disappeared in 10 days. None of the specimens showed marked depression and no symptoms of encephalitis were observed. The total leucocyte count was not greatly altered in any of the specimens, but there was probably a significant increase of neutrophil cells in all animals except the mature ewe. This mature animal seemed entirely refractory to the listeria cells, except for the transitory vaginitis. Since she had a pre-exposure antibody titer of 1:100 the question of age resistance or immune status was raised here, but on one specimen there could be little more than conjecture. It may be argued favorably, however, that her lack of agglutinin response gives significance to the titers of the specimens which produced agglutinins (example in table 1). These titers probably mean that the antigenic stimulation was intimate within the tissues of the host and not simply the result of antigenic substances placed upon the mucous membranes. If the latter had been the case, we would have expected a titer in the mature animal as well.

One animal was killed after 10 days, at which time listeria cells were found in the tissues of the cervix and vagina (table 2). Another specimen killed 16 days after exposure was bacteriologically negative.

Seventy-nine days after the exposure one sheep was still carrying a titer of 1:400.

*Intragastric exposures.*—To determine the possibilities for ingestion as a mode of transmission, 5 sheep were exposed by depositing listeria cells in the rumen. The dose of 5 billion listeria cells per kg was given by stomach tube. The tube was flushed with water before it was removed from the esophagus, but listeria organisms undoubtedly contacted the structures of the pharynx and oral cavity as the tube was withdrawn. These structures became further exposed when rumen contents were regurgitated in the process of rumination. This was, therefore, a gastrointestinal exposure rather than an exposure limited to some segment of the alimentary tract.

The group consisted of three 11-month-old lambs, a 4-year-old ewe, and her 3-week-old lamb. For 3 days after the exposure the animals showed evidence of depression, hyperthermia, and inappetance. This period was associated with an average weight loss of 5 pounds for the three 11-month-old animals. The growth curve of the baby lamb remained static for 8 days following the exposure. Dyspnea, coughing, and some increase in the nasal secretions were also noted in the first few days following the exposure. The latter effect was reminiscent of the rhinitis reported by Olafson[4] from naturally-occurring cases of ovine listeriosis. No symptoms referable to the central nervous system were noted. The white blood cell response was uniform and rapid in all specimens except the mature ewe (example in table 1). Here again, as in all the instances of leucocytosis in the sheep, a great abundance of immature neutrophil forms was observed. The leucocytes returned to normal values by the 6th day, but this was then followed by a second rise on the 8th day to values greater than 1 standard deviation above the normal mean for all 5 specimens. It was interesting that the leucocytes of the mature ewe did not increase until the occurrence of this second wave of leucocytosis. She reached her peak count on the 9th day, which was also the period of her highest febrile response.

The agglutinins had risen to a 1:50 titer in sheep 325 after 7 days, at which time he was sacrificed to determine the distribution of the bacterial parasite. Autopsy revealed no gross lesions that could be associated with the listeria infection. However, cultures of *L. monocytogenes* were obtained from the liver, spleen, suprapharyngeal and mesenteric lymph nodes, testicle, pharynx, and nasal mucosa (table 2). Histologically the jejunum had an excessive infiltration of mononuclear and polymorphonuclear leucocytes in the lamina propria (fig. 2). Host reaction in this tissue was probably associated with the presence of listeria cells and very likely indicated a site of entry.

Another animal sacrificed 29 days after exposure was negative on bacteriological examination.

The three remaining animals in the group were held for further observation. While the baby lamb reacted to the exposure in a manner that left little doubt of her having been infected, she did not develop agglutinins. This failure to produce agglutinating antibodies was considered to be due to the immaturity of the antibody-forming tissues of the young animal, which rendered it incapable of response to the antigens.

The mature ewe demonstrated little reaction to the listeria cells immediately

following the exposure, but on the 9th day she suddenly developed febrile and leucocyte responses, which were followed 9 days later by the appearance of aggutinating antibodies. The reaction in this individual was suggestive of an incubation period and, as was noted previously, the 9th day was associated with a second wave of leucocytosis in all the other specimens in the group. This second increase in white blood cells was less marked than the original reaction on the first day, but it was nevertheless consistent throughout the group and further strengthens the hypothesis that the cells were emerging from some focus in such a manner as to evoke a host reaction.

One animal was still reacting at a titer of 1:400 after 79 days.

There was considerable evidence that the listeria cells invaded the tissues in all 5 animals exposed by the gastrointestinal route. Positive proof of this was obtained in one animal which was autopsied 7 days after exposure. It is noteworthy that the bacterial cells in this specimen were in lymph nodes that were receiving lymph directly from the alimentary tract and that the organisms were still found in the nasal mucosa and pharynx after 7 days. A generalized distribution of the bacterium within the host was proved by recoveries from liver, spleen, and testicle.

## DISCUSSION

To state that listeriosis resulted from nearly every mucous membrane exposure in the sheep experiments requires some discussion. It would appear that workers studying this bacterium have usually required the development of encephalitis or meningoencephalitis as proof that a disease was reproduced. Such a disease manifestation need not be considered essential for the detection of infection. The concepts of infection and disease need clarification in this disease problem. The experimental sheep were infected, as was demonstrated by a variety of host reactions, and recovery of the infecting agent from some specimens. These latter specimens had demonstrated host responses no different from other members of the various groups, which makes it probable that the nonsacrificed sheep also harbored the bacterium in their tissues at some period following exposure. The data presented would seem to fulfill the requirements of infection. The evidences of clinical disease shown by these animals would very likely have gone undetected if the animals had not been carefully observed. By cursory examination it would be said that disease had not occurred. In the 16 experimental sheep clinical encephalitis, with histopathological changes in the brain and isolation of the bacterium from that tissue, occurred in only one animal. The symptoms noted in that specimen (sheep 1098) were those of stupidity and irresponsiveness. Similar atypical types of clinical syndromes are sometimes seen in field cases. The development of an encephalitic process in such a small percentage of the experimental animals is probably not so disappointing a result as it would appear on first analysis. If encephalitis is regarded as an occasional manifestation of listeria infections, then the fact that only 1 of the 16 exposed animals developed pathological changes in the central nervous system could be attributed to the chance occurrences that take place in the interacting complex biological systems of both the host and parasite. In naturally infected ruminant populations encephalitis commonly occurs in only about 1% of the group, and this figure rarely exceeds 20%. To expect encephalitis from each case of experimental listeriosis would seem to be as untenable as a require-

FIG. 1.—Medulla oblongata of sheep 1098 showing perivascular cuffs of mononuclear leucocytes and edema in the Virchow-Robin spaces. ×200.

ment that each case of bovine brucellosis result in abortion or that each infection by the virus of equine encephalomyelitis progess to an encephalitic process.

The idea that a disease agent producing pathological alterations in the brain should find easy access to that tissue through the intranasal route is an attractive one and often receives first consideration in studies on the pathogenesis of the encephalitides. It would seem that the proximity of the olfactory tracts to the nasal mucosa would make transit of the agent into the brain quite direct and easy. Critical analysis of this hypothesis requires the occurrence of certain specific events. First of all, the agent must invade a relatively small area of nasal mucosa. It must then, in some manner, follow the olfactory nerve into olfactory bulbs and tracts. It would seem reasonable to expect that the rhinencephalon, in the case of a bac-

FIG. 2.—Jejunum of intragastrally exposed sheep (325) showing excessive mononuclear and polymorphonuclear leucocytic infiltrations in the lamina propria. ×200.

terial invasion, should receive the brunt of the pathological alterations and that the agent should be found there but not necessarily anywhere else. If, on the other hand, we could presume that the agent was less specifically neurotropic and impinged elsewhere on the nasal mucosa, it would probably be picked up by the lymphatics and carried thus to the blood, from which it might reach the brain by the hematogenous route. This would necessitate what may be called a generalized infection, and it seems to be the route by which such agents as the meningococcus reach the central nervous system. The possibility of a generalized dissemination of the agent in the body of the host makes extension through the nasal mucosa no longer necessary, since any other parenteral entry into the lymph or blood might serve as well. In the experiments reported in a previous paper,[1] there were 5 instances out of 7 where listeria cells were re-

covered from the brains of rabbits following intranasal instillation. Nevertheless, this is not proposed as the most likely way that clinical listeriosis may become established in nature. In these animals care was taken to make certain that the olfactory area of the nasal mucosa was extensively exposed to the organisms, as were other surfaces of the mucosa. This was followed by an extensive purulent rhinitis, which resulted not only in entry to the brain but to the lymph and blood as well. The severe inflammatory reactions in the nasal cavity were followed by extension of the process into the cranial cavity of the rabbits. An expansion of the process over the meninges then occurred and was associated only occasionally with local areas of encephalitis that were adjacent to severely involved meningeal membranes. This was not analogous to the pathological changes described for most cases of listeriosis in ruminants. The usual pathological picture in listeria encephalitis is one of perivascular cuffing associated with focal infiltrations of leucocytes in the parenchyma of the brain. Such lesions are most commonly located in the brain stem from the cervical cord to the thalamus but may also be found in the cerebellum and cerebellar meninges. Meningitis sometimes accompanies the encephalitis but often appears to be an extension from the brain process.

Sheep 1098 offered interesting possibilities on the pathogenesis of encephalitis. The animal had been exposed intranasally. However, the olfactory bulb did not contain listeria organisms, and the rhinencephalon showed no pathological alterations. The medulla oblongata harbored the organism and was undergoing the early pathological changes seen in natural cases of encephalitis. The authors prefer to think that the bacterial cells reached the nervous tissue by way of the blood, even though the original infection was in the nasal mucosa.

The preponderance of fatal listeria infections in sheep occurs in the winter and early spring months, when the ewes are usually lambing. This association of the disease with animals that are in late pregnancy and with lambs and ewes a short time after parturition suggests that the genital tract with its gravid uterus may have significance in the annual periodicity of the disease. This possibility is further strengthened by the isolation of *L. monocytogenes* from cases of abortion in various species of animals.[2,7–10]

The results of the experiments in the intravaginal group do not preclude the possibility that listeria organisms may find the environment of the placenta or the endometrium favorable for their growth, and develop there during the gestation period if the ewes harbor focal areas of infection. The possibility also exists that listeria cells deposited in the genital tract at the time of coitus might find the environment desirable and use the developing tissues as a medium, thus causing occasional death of lambs by abortion and encephalitis. The testicle of one of the intragastrally exposed sheep contained listeria organisms.

7. Paterson, J. S. 1940, Studies on organisms of the genus *Listerella*, IV—An outbreak of abortion associated with the recovery of *Listerella* from the aborted foetuses. Vet. J. **96:** 327–332.
8. Van Dorssen, C. A. and Janson, J. 1951, Over Het Practische Belang Van *Listeria*-infectie. Tijdsche. voor Diergeneesk. **76:** 756–767.
9. Levi, M. L., Shamir, A. and Neeman, G. 1952, Two cases of bovine abortion associated with *Erysipilothrix* (*Listeria*) *monocytogenes*. Refuah Veterinarith (Vet. Med.) Quarterly of Israel Vet. Med. Assoc. 9.
10. Eveleth, D. F., Goldsby, A. I., Bolin, F. M. Holm, G. C. and Turn, J. 1953, Epizoology of vibriosis and listeriosis of sheep and cattle. Vet. Med. **48:** 321–323.

Further work would be desirable to determine whether the male gonad might be a site for persistent residence of the parasite, as is known to occur in brucella infections of cattle and swine.

The gastrointestinal tract may very likely be the commonest portal of entry for listeria organisms in natural infections. The results of these experiments in both sheep and rabbits have shown that invasion and wide dissemination may occur through these tissues. It is not possible to say which segment of the gastrointestinal tract was penetrated in the establishment of the parasite within the host, since bacterial cells were found at autopsy in both suprapharyngeal and mesenteric lymph nodes. Certainly there may be opportunities for ingestion of these cells if from the shedding of listeria organisms by the experimental rabbits it can be inferred that small wild mammals may act as reservoirs. The passage of listeria cells into the urine or intestinal tracts of rabbits raises the question of rodent contamination of the feed and water of domesticated animals. Clinical listeriosis most often occurs at periods when animals are congregated. Examples of this are outbreaks in feed-lot cattle and in sheep at lambing time. Such stresses on the animals may function as provocative stimuli for susceptibility to infection. A concentration of animals is usually concurrent with a concentration of feed. Rodents and other small mammals are commonly present in such a situation.

The riddle in experimental studies with *L. monocytogenes* in ruminants has been to find a way of reproducing the encephalitic disease. Numerous routes of inoculation of the bacterium have been tried, such as intracarotid, intracerebral, intravenous, subcutaneous, intradermal, intramuscular, etc.[2,6,11] Intracerebral and intracarotid inoculations are usually successful, while those made intravenously are occasionally successful. Other methods usually fail. This seems less like an enigma if one considers that the experimentalist wants to establish brain invasion with each injection, whereas in nature certain chance happenings are probably needed to procure that result. The inference from all of these inoculations and from the results of the present investigation is that the clearance of this organism by the host is quite effective. If the clearance mechanisms are avoided, as is done by intracarotid injection, a great number of cells are provided direct transit to the blood vessels of the brain. Contrasted to this, if the cells enter the venous blood, subcutaneous tissues, etc., they must follow pathways which will bring them in contact with several clearance tissues. It seems likely that this bacterium can only occasionally avoid screening by these mechanisms in adequate numbers to establish brain lesions.

It is tempting to theorize that the rapidly invasive characteristics of listeria cells favor their chances of entering the parenchyma of the brain. As the bacterial cells are carried by the body fluids, they may be attempting to establish themselves in various structures contacted along the vascular pathways. It probably takes a combination of numbers, individual host susceptibility, and freedom from host interference for them to establish a new focus of infection. One might infer from the scant lesions in most body tissues of ruminants, except the brain, that these hosts are relatively refractory to listeria cells and quite rapidly destroy them. However, if by chance the bacteria reach the

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11. Olson, C. Jr., Bagdonas, V., Rollins, C. L. and Blore, I. C. 1953, The relation of silage to listeriosis in sheep. Am. J. Vet. Res. **14**: 202–208.

brain, they enter an environment that may be freer from the processes of host resistance, such as opsonization. This might permit colonization of the bacteria for a time, since the organisms have been demonstrated in the neutrophilic aggregations found in the brain.[4,12]

Five of the nine sheep autopsied in this study yielded listeria cultures. These animals were killed 6 to 15 days after their contact with the organism. Four of the five positive animals were infected by mucous membrane exposures. The original culturing procedure on these animals yielded only 1 positive culture, while reculture 45 days later yielded a total of 19 isolations. The 4 sheep that were bacteriologically negative were sacrificed 16 to 29 days after contact with listeria cells.

Undoubtedly, progress in the understanding of the pathogenesis of listeriosis has been hindered by difficulties in obtaining positive cultures from fresh tissue. Gray et al[13] in 1948 discussed the difficulties in obtaining positive cultures from the bovine brain and described a method for culturing *L. monocytogenes* from brain tissue. These authors refrigerated fresh brain tissue, which was suspended in tryptose broth for a period of 5 weeks to 3 months, and then recultured it. Of 5 positive brain cultures, they obtained the organism on initial examination from only 2. As explanations for this effect they postulated the possibilities of some unstable inhibitory substance in the bovine brain, the release of bacteria by further tissue disintegration, and bacterial multiplication at 4 C.

In the experiments described here and in a previous paper[1] it was considered that brain tissue, though it had shown the phenomenon of listeria bacteriostasis, should present fewer difficulties than other tissues, since the brain seems to be the one organ in these animals where lesions occur consistently, thus indicating probable multiplication at that site. Consequently, all tissues and body fluids cultured in this work were recultured 45 days later. The results leave one fact eminently clear, namely, that negative cultural results from fresh tissues obtained by currently employed means cannot be considered definitive in establishing a diagnosis. It should be recalled here that in the rabbit experiments 79 tissues were positive initially, while 148 were positive on reculture. One sheep tissue was positive on initial culturing, while 19 tissues were positive on reculture. At this writing it can only be reported that this does occur, since the mechanisms for the effect are not known.

If the reculture method is employed in the routine diagnosis of clinical listeria infections, it might be expected that the bacterial cells will frequently be found systemically disseminated and not confined to the brain, as is conventionally thought. Furthermore, it seems reasonable to apply this information in unraveling the etiologies of infectious mononucleosis in man. Seven cases of human illness diagnosed as infectious mononucleosis by three different groups of investigators have yielded *L. monocytogenes* on bacteriological examination.[14] This suggests that the association of the bacterium with the disease has

12. Jungherr, E. 1937, Ovine encephalomyelitis associated with *Listerella* infection. J.A.V.-M.A. **91**: 73–87.

13. Gray, M. L., Stafseth, H. J., Thorp, F., Jr., Sholl, L. B. and Riley, W. F., Jr. 1948, A new technique for isolating listerellae from the bovine brain. J. Bact. **55**: 471–476.

14. Girard, K. F. and Murray, E. G. D. 1951, *Listeria monocytogenes* as the cause of disease in man and animals, and its relation to infectious mononucleosis from an etiological and immunological aspect. Am. J. M. Sc. **221**: Ser. 2: 343–352.

occurred too many times to be explained simply by fortuitous circumstances. The possibility that biopsied lymph nodes may contain undetected listeria cells in some conditions diagnosed as infectious mononucleosis may be considered.

The infection chain linking man with this bacterial parasite has not been adequately explained. One interesting fatal case in a farmer strongly suggested animal-to-man transmission, since a sheep died from listeria encephalitis on the same premises 2 months after the human infection.[15] However, in many cases of listeria meningoencephalitis in man there has been no obvious animal contact by the stricken individuals. The opportunity for meat products to reach the consumer public while harboring listeria organisms seems to exist. The inference from these investigations is that the greater percentage of animals in an involved herd will experience inapparent infections that would not affect their commercial value. If these animals were slaughtered for meat at such a time, the opportunity for man to contact the bacterium could be created.

15. Odegaard, B., Rönnaug, G. and Henriksen, S. D. 1952, A case of *Listeria* infection in man transmitted from sheep. Acta med. Scandinav. **142**: 231–238.


## SUMMARY

1. Instillation of *Listeria monocytogenes* on the nasal mucosa, vagina or gastrointestinal tract of sheep resulted in listeriosis. Evidence of infection became apparent when several host reactions other than encephalitis were studied.

2. Listeria organisms were recovered from the brains of 2 sheep (13%).

3. *L. monocytogenes* was cultured from the tissues of 5 sheep sacrificed 6 to 15 days after exposure. The bacterium was not recovered from 4 sheep sacrificed 16 to 29 days after exposure.

4. A variety of tissues failed to reveal listeria cells on original culturing. Of 19 positive tissues, 18 (95%) were positive only after reculture following a holding period.

# THE EFFECT OF ULTRASONIC IRRADIATION ON CERTAIN PROPERTIES OF INFLUENZA VIRUS

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In addition to employing ultrasonics as a means of disintegrating various bacteria for purposes of liberating enzymes[1] or for extracting and concentrating bacterial cell constituents,[2–7] several of the animal and plant viruses have been subjected to ultrasonic irradiation in order to reduce aggregation or to bring about inactivation.

In 1934, Stanley[8] exposed tobacco mosaic virus to high frequency sound vibrations and found that impure virus was inactivated while purified virus retained its infectivity. Scherp and Chambers[9] were unable to inactivate the viruses of poliomyelitis, human influenza and swine influenza by exposure to 9000 cycles per second for 1 hour. However, by treating the virus sample at a frequency of 450,000 cycles per second and increasing the intensity of the acoustic input, other investigators[10] were able to accomplish the inactivation of poliomyelitis virus. Hamre[11] achieved almost complete inactivation of the influenza virus, type A, by treatment for 1 hour at 700,000 cycles per second.

Preliminary work indicated that certain effects of ultrasonic irradiation of influenza virus, such as inactivation, are influenced not only by acoustic input and time of exposure, but also by the physical or physicochemical associations of the virus with tissue components, the suspending medium and temperature. Accordingly, in the present study, influenza virus, existing in chorioallantoic fluid and as eluted virus, was subjected to the same ultrasonic energy but at various temperatures and times of exposure. Infectivity, hemagglutinating properties and antigenic structure, as measured by hemagglutination-inhibition tests, were examined. Furthermore, the efficiency of ultrasonic vibrations as a technique for liberating influenza virus from chorioallantoic tissue was tested.

## MATERIALS AND METHODS

*Virus.*—The type A (PR8 strain) influenza virus employed in this investigation was in the form of chorioallantoic fluid from infected, embryonated chicken eggs. The virus was pooled

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11. Hamre, D. 1949, J. Bact. **57**: 279–295.

from several passages and was stored at −50 C immediately after harvesting.

*Preparation of eluted virus.*—Equal volumes of a 1:40 saline dilution of chorioallantoic fluid containing virus and a 3% suspension of thrice washed human group 0 erythrocytes were mixed at 4 C for 90 minutes. The mixture was then centrifuged at 2000 r.p.m. for 10 minutes and the supernatant fluid discarded. The cells were resuspended in sterile saline and incubated at 37 C in a water bath for 45 minutes, followed by centrifugation for 10 minutes at 2000 r.p.m. The supernatant fluid was removed, tested for hemagglutinability and stored at −50 C.

*Inoculation and harvesting of virus.*—The routine inoculation of virus into the chorioallantoic sac of 8- to 10-day embryonated hens' eggs was accomplished by introducing 0.1 ml of virus preparation from a tuberculin syringe through a small slit previously cut in the shell with a cutting disc. After inoculation, the shells were sealed with sterile vaspar and placed in a 37 C incubator. When it was necessary to introduce the inoculum onto the chorioallantoic membrane, the procedure employed was essentially the same as that suggested by Beveridge and Burnet.[12]

After 48 hours' incubation, the eggs were candled and those containing viable embryos were chilled at 4 C for at least 2 hours. The shell and shell membrane in the area of the air sac were removed aseptically, and the chorioallantoic fluid was withdrawn by means of a needle and syringe. The pooled fluid was centrifuged at 2000 r.p.m. for 10 minutes, and the supernatant fluid was titrated by the hemagglutination technique. The material was tested for bacterial contamination by inoculating 0.5 ml into thioglycollate broth, with subsequent incubation at 37 C.

Chorioallantoic membranes were collected by removing the shell and shell membrane from above the artificial air sac and aseptically excising the membrane from the remainder of the embryo. After washing twice with sterile saline (buffered to pH 7.4), a 10% saline suspension of membranes by weight was prepared. The virus was liberated from the membranes either by ultrasonic irradiation or by grinding in a mortar with sterile alundum.

*Hemagglutination and hemagglutination-inhibition tests.*—The procedures for the hemagglutination and hemagglutination-inhibition tests were principally the same as those adopted by the United States Army in 1951.[13] Phosphate-buffered physiologic saline (pH 7.4) was employed as the diluting fluid.

*Determination of $EID_{50}$.*—Serial 10-fold saline dilutions of virus were prepared. Two-tenths ml of the desired dilutions was inoculated into the chorioallantoic sac of each of 4 embryonated eggs. After 48 hours' incubation at 37 C, the chorioallantoic fluid of each egg was tested for its hemagglutinating properties. From this, the 50% egg infectivity dose ($EID_{50}$) was calculated by the method of Reed and Muench.[14]

*Immunization of rabbits.*—Rabbits, each weighing approximately 2500 g, were bled by cardiac puncture and the serums recovered in the usual manner. These serums served as controls in the serologic tests. Each animal received 3 intraperitoneal injections of a virus preparation (each injection 1.0 ml) at weekly intervals and were bled from the heart 10 days after the last injection. All serums were inactivated at 56 C for 30 minutes and stored at deep freeze temperature.

*Ultrasonic treatment.*—The ultrasonic generator employed in this investigation was constructed by Battelle Memorial Institute of Columbus, Ohio, and made available through their courtesy. In the generator, a quartz crystal with a fundamental frequency of 450 kc was mounted in a specially designed transducer. The supporting elements consisted of two brass rings which sandwiched the crystal. The lower surface of the crystal was exposed to a column of air. This arrangement resulted in maximum unidirectional transmission of ultrasonic energy from the upper surface of the crystal, since the acoustic reflection factor of air is about 96%. The entire transducer was immersed in oil, which was cooled by being pumped through an ice-water bath. In order to obtain effective heat dissipation, the cooled oil was passed directly across the face of the crystal. Electrical energy of 2500 volts at 0.3 ampere (750 watts) was employed for the treatments. Approximately 60% of this energy was delivered to the transducer. The treatment chamber consisted of a double-walled steel container which permitted cooling of the sample during exposure, since chilled air could be circulated between the two walls. The temperature of the sample could

12. Beveridge, W. I. B. and Burnet, F. M. 1946, Med. Res. Council Special Rep. Ser. no. 256, His Majesty's Stationery Office, London, England, pp. 14–16.

13. 1951, Methods for Medical Laboratory Technicians, Departments of the Army and the Air Force TM 8-227—AFM 160-14, Washington, U. S. Government Printing Office, pp. 523–558.

14. Reed, L. J. and Muench, H. 1938, Am. J. Hyg. **27:** 493–497.

be determined at intervals by means of a thermocouple.

Twenty-five ml of sample were treated during a single exposure. Penicillin and streptomycin were added to all samples of virus in the quantities of 600 units and 1.25 mg per ml of sample, respectively. These dosages were found by Davenport and Francis[15] to be adequate for minimizing bacterial contamination.

## EXPERIMENTAL RESULTS

*1. The effect of varying the time of ultrasonic treatment on the infectivity and hemagglutinability of influenza virus when temperature was held constant.*— It was previously observed that the temperature in a virus sample rose quite rapidly during the course of exposure. In order to determine the influence of time of treatment on egg infectivity and hemagglutinability of the samples, the temperature was not permitted to exceed 30 C. This was accomplished as described above.

Infected chorioallantoic fluid and eluted influenza virus were subjected to ultrasonic vibrations for 30 minutes. At 5 minute intervals, 1.5 ml were withdrawn from each sample and stored for future infectivity and hemagglutination tests. As shown in table 1, there was no alteration in titers of samples studied throughout the 30 minutes of treatment, regardless of the state of the virus.

*2. The effect of heightened temperature during exposure of virus to ultrasonics.*— Since treatment of virus at temperatures below 30 C had no apparent effect as measured by the aforementioned criteria, samples of crude and eluted virus were treated under conditions similar to those stated above, except that no attempt was made to control the temperature of the samples until it exceeded 50 C. A preliminary study had revealed that short exposures of the virus preparations to this temperature did not result in a decrease in the $EID_{50}$ or the hemagglutinating capacity of the samples.

TABLE 1.—*Influence of time and constant temperature of ultrasonic treatment on influenza virus.*

| Ultrasonic Treatment (in minutes) | Chorioallantoic fluid | | Eluted virus | |
|---|---|---|---|---|
| | Hemagglutination titer | $EID_{50}$ | Hemagglutination titer | $EID_{50}$ |
| 0 (control) | 1:1024 | $10^{-7.1}$ | 1:128 | $10^{-6.4}$ |
| 5 | 1:1024 | $10^{-7.1}$ | 1:128 | $10^{-6.7}$ |
| 10 | 1:1024 | $10^{-7.5}$ | 1:128 | $10^{-6.6}$ |
| 15 | 1:1024 | $10^{-7.0}$ | 1:128 | $10^{-6.2}$ |
| 20 | 1:1024 | $10^{-6.9}$ | 1:128 | $10^{-6.4}$ |
| 25 | 1:1024 | $10^{-7.0}$ | 1:128 | $10^{-6.2}$ |
| 30 | 1:1024 | $10^{-7.4}$ | 1:128 | $10^{-6.7}$ |

Influenza virus, as chorioallantoic fluid, was treated for 5 minutes. The temperature of the material was 21 C at the beginning of the exposure, and by means of a thermo-couple, temperature readings were made every 30 seconds during the treatment period. During the 5 minute exposure, the temperature rose to 50 C. The temperature of the eluted virus sample, measured under the same conditions, rose to 50 C within 2.5 minutes at which time treatment was stopped, the virus cooled to 21 C in an ice bath and the exposure continued for an additional 2.5 minutes. The increase in temperature of the samples plotted against time is shown in figure 1.

Control samples of virus were subjected to the same conditions of temperature, but without ultrasonic treatment. After 5-minute exposure, the treated virus preparations and the control samples were tested for their hemagglutinability and infectivity. Although the increase of temperature had no effect on the hemagglutinating properties of the two virus preparations (see table 2), the $EID_{50}$ of the eluted virus was reduced from $10^{-7.5}$ to $10^{-3.4}$. On the other hand, the infectivity of the chorioallantoic fluid was relatively unaffected.

15. Davenport, F. M. and Francis, T., Jr. 1951, J. Exper. Med. **93**: 129–137.

TABLE 2.—*The effect of heightened temperature during exposure of virus preparations to ultrasound.*

| Ultrasonic treatment (in minutes) | Hemagglutination titer | $EID_{50}$ |
|---|---|---|
| | Chorio-allantoic fluid | |
| 0 (control) | 1:1024 | $10^{-6.0}$ |
| 5 | 1:1024 | $10^{-6.5}$ |
| | Eluted virus | |
| 0 (control) | 1:128 | $10^{-7.5}$ |
| 5 | 1:128 | $10^{-8.4}$ |

*3. Ultrasonic irradiation of virus-infected chorioallantoic membranes.*—In the previous experiments the virus preparations exposed to ultrasonic energy were in fluid menstrum. Infected chorioallantoic membranes have been used as a source of influenza virus, but procurement of the virus has depended on the disruption of the tissue. The conventional methods of achieving this have been hand-grinding and homogenization in a blendor. Since treatment of virus preparations at temperatures lower than 30 C did not alter the infectivity or hemagglutinating capacities of the samples, the efficacy of ultrasound as a means of liberating virus from tissue was tested.

Infected chorioallantoic membranes were harvested, pooled, suspended in saline (10% by weight) and ground in a mortar as described previously. After removing gross particles by centrifugation, a sample of the supernatant fluid was removed for testing and the remainder was exposed to ultrasonic energy for 5 minutes. The temperature during ultrasonic treatment was not permitted to exceed 30 C. A similar suspension of membranes was exposed directly to ultrasonic irradiation without benefit of preliminary grinding. After 1.0 minute of exposure, a 1.5 ml aliquot of suspension was removed and centrifuged as before. At the end of 5 minutes, the procedure was repeated. The results of hemagglutination and infectivity tests of all samples are recorded in table 3.

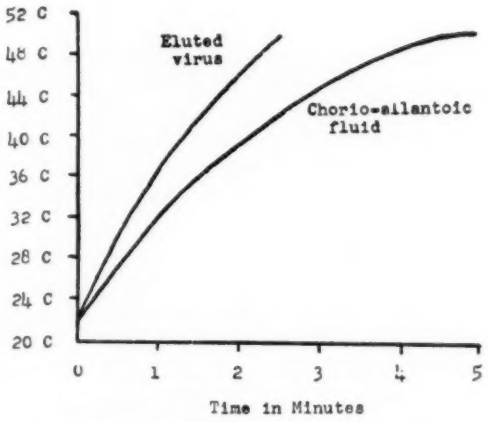


FIG. 1.—The influence of ultrasonic irradiation on temperature of eluted virus and virus as chorioallantoic fluid.

Ultrasound is as efficient a method of liberating influenza virus from infected membranes as is manual grinding. Exposure of supernatant fluid, following grinding, to ultrasound for 5 minutes did not alter its infectivity or hemagglutinability. Furthermore, virus is liberated quite rapidly by ultrasonic vibrations, since there is no quantitative difference in the amount of virus liberated after 1 minute of treatment as compared with the amount liberated after 5 minutes.

*4. Effect of ultrasonics on antigenicity of influenza virus as determined by hemagglutination-inhibition tests.*—In order to test the effect of ultrasonic vibrations on the antigenicity of treated virus, antiserums were obtained from rabbits which had received immunizing doses of samples of eluted virus, vari-

TABLE 3.—*The efficiency of ultrasonic vibrations in liberating influenza virus from chorioallantoic membranes.*

| Treatment of membranes | Hemagglutination titer | $EID_{50}$ |
|---|---|---|
| Grinding in mortar | 1:5121 | $0^{-6.9}$ |
| Grinding followed by 5 minute exposure of supernatant fluid to ultrasound | 1:512 | $10^{-6.7}$ |
| Membranes treated with ultrasound for 1 minute | 1:512 | $10^{-6.8}$ |
| Membranes treated with ultrasound for 5 minutes | 1:512 | $10^{-6.6}$ |

TABLE 4.—*Effect of ultrasonic irradiation on antigenicity of eluted virus as measured by hemagglutination-inhibition.*

| | Serum 1 | Serum 2 | Serum 3 | Serum 4 |
|---|---|---|---|---|
| Unheated and untreated virus | 1:512 | 1:512 | 1:1024 | 1:1024 |
| Untreated virus, heated to 50 C | 1:512 | 1:1024 | 1:1024 | 1:512 |
| Treated virus, temperature less than 30 C | 1:512 | 1:1024 | 1:1024 | 1:1024 |
| Treated virus, temperature at 50 C | 1:512 | 1:1024 | 1:512 | 1:1024 |
| Infectious chorioallantoic fluid, unheated and untreated | 1:512 | 1:512 | 1:1024 | 1:1024 |

Serum 1: antiserum to unheated and untreated virus.
Serum 2: antiserum to untreated virus, heated to 50 C.
Serum 3: antiserum to treated virus, temperature less than 30 C.
Serum 4: antiserum to treated virus, temperature to 50 C.

ously treated as listed in table 4. Eluted virus was chosen as the test agent, since it will be recalled that temperature of 50 C decreased the infectivity of this material after 5 minutes' exposure to irradiation.

Hemagglutination-inhibition tests were performed with both the homologous and heterologous systems. None of the serums procured prior to immunization produced inhibition. The results of these tests (table 4) demonstrate that the types of treatment rendered the eluted virus did not alter the antigenic properties of the virus as measured by this technique.

## DISCUSSION

In the past, it has been shown that the fluid medium in which virus is suspended and the presence of extraneous material affect the results of exposure to ultrasonic vibrations. Rivers et al[16] and Yaoi et al[17] reported that impurities prevent the inactivation of vaccinia virus suspension by ultrasonic irradiation. Conversely, Stanley[8] found that impure tobacco mosaic virus could be inactivated by ultrasound, while purified virus was unaffected. In the studies just described the egg infectivity of eluted virus was decreased if treated at 50 C, while the infectivity of the virus in the form of chorioallantoic fluid remained unchanged. Although these results are not in agreement with those of Stanley,[8] mention should be made of several factors which play important roles in determining the effects of ultrasonics.

Apparently there is general agreement that the phenomena resulting from the exposure of biologic material to ultrasonic vibrations are associated with cavitation. Acoustic energy below the cavitation level results in little or no effect.[18,19] When a liquid is subjected to ultrasonic vibrations, areas of negative pressure are produced. In these small areas, cavitation bubbles of dissolved gases are formed. As these bubbles move into an area of positive pressure, they collapse (implode) and tremendous forces are exerted locally. It is likely that these shock waves are responsible for the various phenomena brought about by ultrasonic irradiation. Frequency of oscillation is thought to be of considerably less importance than acoustic intensity in determining the effects of ultrasonics on biologic systems. It appears that there is no straight line relationship between frequency and the effects of ultrasound.[1,20] The amount of dissolved gases in the biologic suspending medium also affects the acoustic

16. Rivers, T. M., Smadel, J. E., and Chambers, L. A. 1937, J. Exper. Med. **65**: 677–685.
17. Yaoi, H. and Nakahara, W. 1934, Jap. J. Exper. Med. **12**: 131–135.
18. Elpiner, I. E. 1952, Ultrasonic waves in microbiology (Russian) Mikrobiolgiya, **21**: (2) 228–238.
19. Horton, J. P. 1951, Doctoral thesis, The Massachusetts Institute of Technology.
20. Newton, N. 1953, Battelle Memorial Institute, Columbus, Ohio. Personal communication.

power level at which cavitation occurs and is therefore intimately associated with ultrasonic phenomena. When the gas content of the medium is comparatively high, cavitation occurs at much lower energy levels than when the suspending medium is essentially free of gas. Horton,[19] as well as others, has stated that surface tension markedly influences the results of exposing biologic materials to ultrasound. A lowering of surface tension decreases the cavitational effects produced by acoustic energy.

The significance of structure and size in relation to sonic susceptibility has been reported by Anderson, Boggs and Winters,[21] who found that the larger T phages were sonically susceptible while the smaller phages were resistant. It would seem possible, other things being equal, that cavitation bubbles would occur with greater frequency on the surface of a larger particle than on the surface of a smaller one. Thus the frequency of cavitation and the size of the particle would influence the results of exposure.

Undoubtedly the factors mentioned thus far are reflected in the findings reported in this study as well as others. It is quite possible that chorioallantoic fluid and saline differ in their capacities as coupling mediums for acoustic energy. This capacity, in turn, is associated, at least in part, with the factors mentioned above.

The importance of temperature is quite obvious since the temperature of eluted virus rose to 50 C in half the time required for infected chorioallantoic fluid. Accompanying this temperature rise was a decrease in infectivity, which was not noted if the sample was cooled to below 30 C during the exposure. Since acoustic energy is converted to heat energy, the physical properties of the suspending mediums (chorioallantoic fluid and saline) differ in their heat-dissipation capacities. Since it is well recognized that extraneous proteins exert a buffering action against denaturation by heat, the saline environment of the eluted virus must be considered as playing an influential role in the loss of infectivity mentioned above. As the internal temperature of the eluted virus increases, the amount of energy necessary to rupture intermolecular bonds decreases, and thus a given acoustic energy produces more cavitation. A greater resistance of the hemagglutinating moiety of the eluted virus to cavitational effects would account for the persistence of this property under conditions which resulted in a decrease in infectivity. Therefore, for any particular biologic system, the energy-temperature relationship must be carefully considered.[19]

Furthermore, the rapidity with which ultrasonic energy might affect biologic systems under proper conditions is emphasized by the liberation of virus from infected chorioallantoic membranes within 1 minute. These amounts were equal to those obtained after 5 minutes of treatment or by manual grinding. These results suggest the possible application of ultrasonics for the liberation of virus from cellular material.

The results of these experiments indicate that ultrasonic energy may be used to liberate virus from infected tissue and that a certain combination of ultrasound and temperature produced a synergistic effect which inactivated the virus without modifying its antigenic and hemagglutinating properties. Should it be possible to recognize the parameters which function in the application of ultrasonics to biologic systems, it might be well to explore this technique further as a means of over-

21. Anderson, T. F., Boggs, S. and Winters, B. C. 1948, Science, **108**: 18.

coming several of the difficulties met in the preparation of virus vaccines, among which are the release of virus from infected tissue and subsequent inactivation without antigenic modification. Such findings become more pertinent in view of new advances in ultrasonics engineering which indicate the practicality of devices and methods for large-scale treatment.

## SUMMARY

1. Influenza virus in chorioallantoic fluid was not inactivated by ultrasonic treatment at temperatures of less than 30 C and at 50 C.

2. Influenza virus, partially purified by elution from erythrocytes into saline, demonstrated a decreased infectivity when treated at temperatures rising to 50 C. This inactivation was not accompanied by apparent changes in antigenicity nor was there loss of hemagglutinability.

3. Temperature readings conducted while ultrasonically treating infectious chorioallantoic fluid and eluted virus revealed that the internal temperature of the latter preparation reached 50 C in half the time of exposure required for the chorioallantoic fluid.

4. Liberation of virus from chorioallantoic membranes was accomplished by ultrasonic irradiation within 1 minute. The quantity of virus freed was equal to that obtained by grinding and was not increased by ultrasonic exposure for 5 minutes.

# ANTI-EGG PRECIPITINS IN THE SERUM OF HUMANS INFECTED WITH *SCHISTOSOMA MANSONI*

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The serums of human and experimental animals infected with *Schistosoma mansoni* have been shown to have an in vitro effect against the cercarial form of this parasite. Vogel and Minning[1] observed a homogenous membrane which formed around individual cercariae; Papirmeister and Bang[2] and Liu and Bang[3] described precipitate around cercariae and their agglutination when incubated in immune serum. The agglutinins were present in high titer and caused tight clumping of the cercariae.

Since three morphologically distinct forms of the parasite occur in man—metacercariae, adults and eggs—it is of academic interest to determine whether stage-specific antibodies are produced by the host, the time of their appearance and the permanence of such antibodies; it is of practical importance for improving the diagnostic and prognostic tools available in this disease.

Schistosome eggs lodge in the host's tissues for long periods of time and may, therefore, be a potent and constant source of antigen leading to the formation of antibodies qualitatively different from those developing against the cercariae and the adults. Studies herein reported reveal that there is a specific response against egg antigen manifested by an antibody causing in vitro precipitation around the egg.

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1. Vogel, H. and Minning, E. 1948. Hüllenbildung bei Bilharzia-Cercarien im Serum bilharzia-infizierter Tiere und Menschen. Zentralbl. f. Bakt., I, Orig. **153:** 99–105.
2. Papirmeister, B. and Bang, F. B. 1948, The *in vitro* action of immune serum on cercariae of *S. mansoni*. Am. J. Hyg. **48:** 74–80.
3. Liu Ch'ien and Bang, F. B. 1950, Agglutination of cercariae of *S. mansoni* by immune sera. Proc. Soc. Exper. Biol. & Med. **74:** 68–72.

## MATERIAL AND METHODS

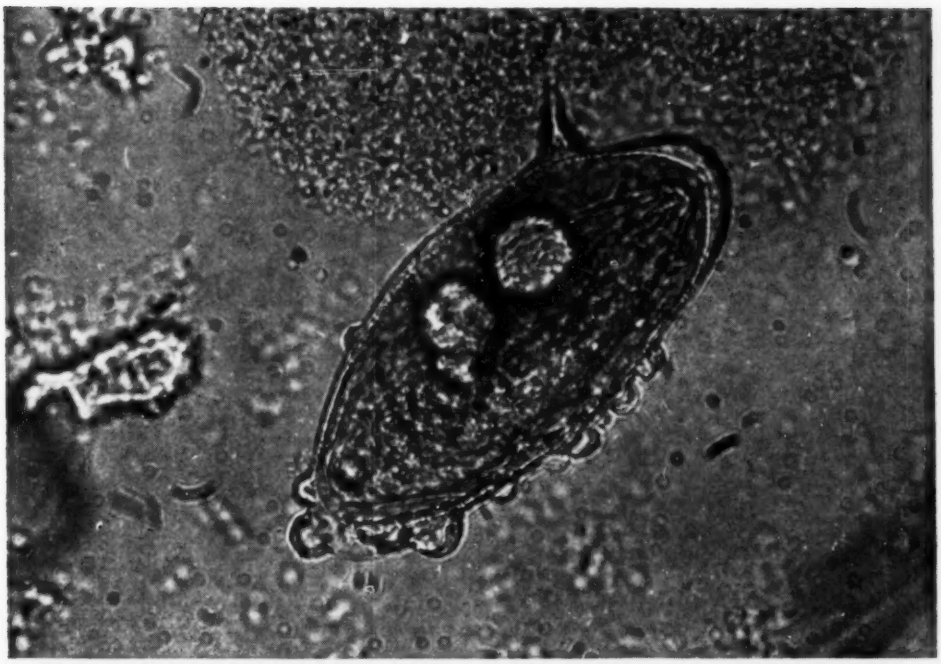
The schistosome eggs utilized in this study were obtained from the livers of mice exposed to cercariae of *S. mansoni* for a period of at least 8 weeks. The livers of freshly killed mice were placed in tap water and minced finely by means of a Waring blendor. The suspension was run through 50 and 100 mesh sieves into a conical sedimentation glass, and allowed to sediment for at least 15 minutes. The sediment was removed with a capillary pipette and placed in a separate glass to which 0.85% sodium chloride solution was added in order to delay hatching. This egg suspension, containing some liver substance, was washed several times in saline and then stored in the refrigerator to be used on the same day. Four livers of heavily infected mice usually produced a suspension containing 75 to 100 eggs per 0.05 cc.

Eggs of hookworm, *Ascaris* and *Trichiuris*, were obtained from washed and sedimented feces, and those of *Fasciola hepatica* were recovered as discharged from adult flukes kept in distilled water.

Positive serums were obtained from 34 Puerto Rican adults and children who were known to have been infected with *S. mansoni* by previous positive stool examinations. Control serums were obtained from continental Americans who presumably had never been exposed to schistosomiasis, and from Puerto Rican children known to harbor *Ascaris*, hookworm, *Trichiuris* and *Endamoeba histolytica*, but free from *S. mansoni*, based on repeated fecal examinations using concentration techniques.

Animal serums were obtained from experimentally infected monkeys and from rabbits immunized by injection of lyophilized schistosome egg material.

Absorption of antibodies from serums was attempted, using either lyophilized or live worm materials. To small volumes of serum inactivated at 56 C for 30 minutes, enough lyophilized material was added to make a 1% suspension. The



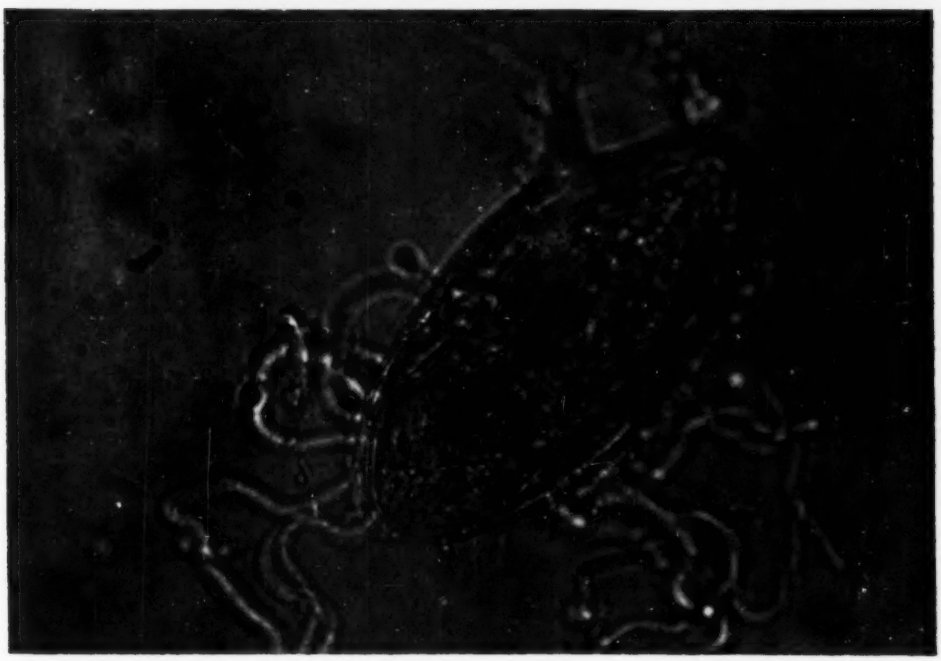


FIG. 1 (top).—Egg of *S. mansoni* with globular precipitate (2+) after incubation in serum of infected humans for 24 hours. ×430.

FIG. 2 (bottom).—Egg of *S. mansoni* with precipitate in long chains (4+) after incubation in serum from experimentally infected monkeys. ×430.

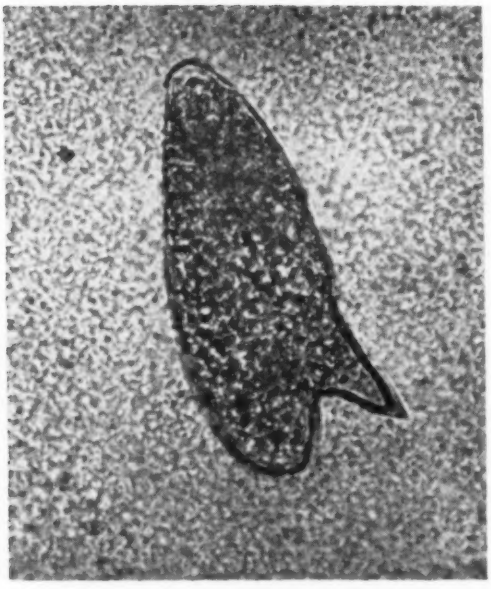


FIG. 3.—Disintegrated egg unaffected by incubation in serum of infected human. ×430.

mixture was incubated at 37 C for 1 hour, centrifuged at 4000 r.p.m. for 5 minutes; the supernatant was then removed and treated again with fresh worm material. The serum was absorbed three times with incubation for 1 hour at 37 C; after the third incubation the mixture was held in the refrigerator at 6 C for 18 hours before centrifugation. The final supernatant was used as the absorbed serum. Absorption with living material was performed in a similar manner, using known numbers of the parasitic forms in saline. Before adding the serum, the living material was centrifuged at 4000 r.p.m. for 10 minutes and the supernatant saline discarded. The serum to be absorbed was then added and adult worms or eggs broken up with the aid of a stirring rod.

For the test, 0.05 of serum was added to an equal volume of the egg suspension on a slide. The mixture was then sealed with a cover glass rimmed with vaseline, the slides incubated at 37 C for periods up to 24 hours, and searched under low power of the microscope for eggs showing precipitate or other reactions. The percentage of total eggs examined which showed precipitate around the edge of the egg shell was recorded.

## RESULTS

When schistosome eggs were incubated in serums from infected humans or monkeys, or artificially immunized rabbits, a precipitate appeared contiguous with the edge of the egg shell in the form of small globules. These globules resembled those formed around the orifices of trichina and ascaris larvae incubated in immune serum.[4,5] The globules varied in size and form, sometimes appearing as single globules; at others forming chains reaching a length of 150$\mu$ or longer, shaped like tape worm proglottids (fig. 1 and 2). Although precipitate formation could be seen as early as 2 hours after incubation, the effect of the serum was best observed after incubation at 37 C for 24 hours.

The serum of all 34 individuals infected with *S. mansoni* caused precipitate formation. The serums of 15 continental Americans were tested and only 2 gave positive reactions; no schistosome ova were found in the feces of these individuals, but both had worked in the laboratory with the cercariae of *S. mansoni* over a long period of time. The serums of 9 multiple parasitized individuals, free of *S. mansoni*, all gave negative reactions; 8 of these were infected with *Ascaris*, 4 with hookworm, all with *Trichiuris*, all with *E. histolytica*. All had one or more additional protozoa.

A pool of positive serums was tested for anti-egg precipitins in serial dilution. Observations were made of the percent of eggs affected, and on the amount of precipitate which formed around a known number of eggs, arbitrarily graded as 4+, 3+, 2+, and + (see fig. 1 and 2). Table 2 shows that

4. Oliver-González, J. 1941, The in vitro action of immune serum on the larvae and adults of *Trichinella spiralis*. J. Infect. Dis. **69**: 254–258.

5. Oliver-González, J. 1943, Antigenic analysis of the isolated tissues and body fluids of the roundworm, *Ascaris lumbricoides* var. suum, J. Infect. Dis. **72**: 202–212.

TABLE 1.—*Percent of* S. mansoni *eggs showing precipitate after incubation at 37 C for 24 hours in serum from infected individuals.*

| Case no. | Duration of infection of mice from which eggs were obtained | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 months | | | | | 10 months | | | |
| | Percent positives in single preparations | | | | Average | Percent positives in single preparations | | | Average |
| 3 | 35.9 | 20.0 | 17.2 | 21.7 | 23.7 | 1.6 | 2.8 | 2.5 | 2.3 |
| 4 | 37.6 | 36.4 | 39.6 | 32.8 | 36.6 | 11.0 | 9.9 | 9.9 | 10.3 |
| 6 | 38.6 | 29.4 | 34.2 | 43.5 | 36.6 | 8.3 | 7.6 | 8.1 | 8.0 |
| 7 | 29.3 | 29.7 | 35.3 | 28.8 | 30.8 | 9.9 | 9.1 | 10.6 | 9.8 |
| 9 | 28.0 | 28.2 | 32.8 | 24.4 | 28.3 | 6.8 | 6.3 | 11.5 | 8.3 |
| 25 | 24.1 | 18.6 | 18.2 | 17.3 | 19.6 | 0 | 1.1 | 1.7 | 0.9 |
| 26 | 23.6 | 26.2 | 26.5 | 20.7 | 24.5 | 2.5 | 3.0 | 6.1 | 3.9 |
| 27 | 17.0 | 14.5 | 20.9 | 19.1 | 15.3 | 5.7 | 4.5 | 3.7 | 4.6 |
| 28 | 20.6 | 15.4 | 14.3 | 15.6 | 16.5 | 4.6 | 5.1 | 4.2 | 4.6 |
| 29 | 25.0 | 26.9 | 26.9 | 22.5 | 25.3 | 1.8 | 2.1 | 3.4 | 3.1 |

the percent of positive eggs as well as the amount of precipitation decreased as the serum was diluted.

The percentage of eggs showing precipitate is quite consistent when eggs of the same source are checked in replicate preparations with the same serum; however, significant differences in the percentage of eggs with precipitate was noted when egg suspensions from mice infected at considerable different dates are used as the antigen. Tests done with eggs from the more recently infected mice gave the highest percentage of positivity (tables 1 and 2).

The eggs which showed the greatest amount of precipitate were those which apparently were least affected by the host and contained a well preserved embryo. Only rarely was precipitate formed around dead and disintegrated eggs, usually vacuolated (fig. 3). The degree of serum positivity varied so that 1.1 to 61.7% of eggs showed the reaction.

Treatment of the serums with lyophilized cercariae and adults, or with living adults, had no measurable effect on the anti-egg antibody; however, treatment of the serums with lyophilized or living eggs produced remarkable reduction in effect on the eggs (table 3).

TABLE 2.—*Effect of serum dilution on sensitivity and intensity of the precipitin reaction (pooled positive human serum).*

| Serum dilution | Percent of positives in single preparations | | | | Average | Intensity of precipitation |
|---|---|---|---|---|---|---|
| 1 | 47.8 | 58.1 | 53.2 | 61.7 | 54.9 | 4+ to 3+ |
| 4 | 30.2 | 27.5 | 24.7 | 28.8 | 27.8 | 3+ to 2+ |
| 16 | 18.9 | 19.6 | 25.0 | 26.7 | 22.5 | 2+ |
| 64 | 9.2 | 8.7 | 8.8 | 9.9 | 9.2 | + |
| 256 | 0 | 0 | 0 | 0 | 0 | |
| 1024 | 0 | 0 | 0 | 0 | 0 | |

No precipitate was formed around the eggs of *Ascaris*, hookworm, *Trichiuris* and of *F. hepatica* when these were incubated in the serums from the individuals infected with *S. mansoni.*

## DISCUSSION

The antigenicity of helminth eggs has previously been demonstrated in that the parenteral injection of ascaris eggs

TABLE 3.—*Percent of eggs with precipitate before and after treatment of serums with living and lyophilized worm materials.*

| Serum no. | Material used for absorption of serum | | | | | |
|---|---|---|---|---|---|---|
| | None | Cercariae | Adults | | Eggs | |
| | | Lyophilized | Alive | Lyophilized | Alive | Lyophilized |
| 1 | 16.6 | 21.3 | 15.1 | 21.1 | 0 | 0 |
| 2 | 40.0 | 33.3 | 39.5 | 34.4 | 3.4 | 5.5 |
| 3 | 33.3 | 37.5 | 34.0 | 39.5 | 2.2 | 0 |
| 4 | 51.2 | 33.9 | 41.3 | 35.7 | 6.7 | 2.1 |

resulted in an immune serum which precipitated a suspension prepared from living eggs stripped from adult ascaris.[5] In schistosoma infections, the presence of eggs in tissue is a normal and prominent part of the disease, and it is therefore to be expected that these eggs, present for prolonged periods of time, might serve as antigens and evoke specific antibodies.

The failure of adult and cercarial tissues to absorb the precipitin described herein and its effective absorption by *S. mansoni* eggs, wet or lyophilized, suggest that this precipitin is evoked by antigenic factors which are present in the egg and not in the other intramammalian forms of the schistosome. The failure of the adult material to effectively absorb this antibody, although eggs can be visualized within the uterus, may be due to the fact that the small number of eggs present within the body of the female represents an inadequate antigen for absorption.

Precipitins were demonstrated in the serums of all individuals with a known past history of schistosome infection. The antibody in the pooled serum from these individuals was still active at a dilution of 1:64, which denotes a fairly high concentration of antibody. Information is not yet available whether this antibody persists in long standing infections after complement-fixing and other antibodies disappear, but if it does, the test may have prognostic value in that the disappearance of these antibodies may represent complete healing with elimination of all egg material from the host.

The antibodies producing the precipitate about the schistosome egg are absorbed by whole egg material, an observation similar to the demonstration that precipitate formed around the orifices of *Trichinella spiralis* larvae can be absorbed by treating the serum with whole larval material.[6] The failure of Thorson[7] to absorb precipitins from the serum of rats infected with *Nippostrongylus muris* may be due to the fact that this investigator absorbed the serums with chemically treated worms or extracts of worms rather than the whole worm material, and in no instance was the lipoid fraction present in the absorbing material.

The reaction was more intense and sensitive when eggs from recent rather than from old mouse infections were used. The precipitate formed about intact rather than disintegrated eggs, suggesting that the antigenic substance is present within the egg—very likely secretions or excretions from the miracidia or the fluid bathing the ciliated embryo. It has been suggested that these secretions from the egg ooze out into the host tissues, exert a cytolitic effect, and are responsible for the formation of micro-abscesses.[8,9] It is very likely, therefore, that these secretions or enzymic substances serve as potent antigens which may be responsible for the development of the anti-egg antibodies.

The failure of precipitate to form around hookworm, ascaris and trichiuris eggs when incubated in the anti-schistosoma serum indicates that this antibody is not evoked by nor will it react with a nonspecific nematode egg antigen. This is also indicated by the negative results obtained with the serums from the individuals infected with

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6 Oliver-González, J. 1941, The dual antibody basis of required immunity to *Trichinella spiralis*. J. Infect. Dis. **69**: 254–270.

7. Thorson, A. E. 1953, Studies on the mechanism of immunity in the rat to *Nippostrongylus muris*. Am. J. Hyg. **58**: 1–15.

8. Faust, E. C. 1946, The diagnosis of schistosomiasis Japonica. Am. J. Trop. Med. **26**: 113–123.

9. Hoeppli, R. 1953, Histopathological aspects of human and animal infections with zooparasites. Proc. Alumni Assoc. Malaya, **7**: 60–80.

intestinal nematodes and protozoa. The failure of reaction with unembryonated *F. hepatica* egg indicates that the antibody may be specific for genus *Schistosoma;* however, this must be confirmed with embryonated eggs since, as suggested, the reaction may be due to the secretions and fluids within the embryonated eggs.

## SUMMARY

1. When schistosome eggs obtained from infected mouse livers were incubated at 37 C in the serum from humans and monkeys infected with *Schistosoma mansoni*, a precipitate was formed around the egg. Precipitate was also formed when eggs were incubated in the serums of rabbits artificially immunized with lyophilized schistosome egg material.

2. Precipitate was not formed when schistosome eggs were incubated in the serum from individuals with a proven negative history of schistosomiasis even though infection with *Ascaris*, hookworm, *Trichiuris* and with *Endamoeba histolytica* was present.

3. The precipitin was absorbed by treating the serums with living or lyophilized eggs, obtained from infected mouse livers, but not by living and lyophilized adults and not by lyophilized cercariae. This suggests that there are antigens within the egg either absent or present in too small concentrations in the other two parasitic forms.

4. It is suggested that the secretions from the mircacidium or fluid bathing the embryo may be the antigenic material responsible for precipitin formation in the host and for the formation of the precipitate in vitro.

5. The possibility of using test antigens prepared from egg material for immunological tests, which may be of diagnostic and prognostic value, is mentioned.

# THE EFFECT OF OXOPHENARSINE ON THE PHOSPHATE METABOLISM OF *TRYPANOSOMA EQUIPERDUM* IN THE RAT

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Moraczewski and Kelsey[1] found that measurable quantities of $P^{32}$ appeared in four chemical fractions of *Trypanosoma equiperdum* after inorganic $P^{32}O_4$ was injected into infected rats at a dose of 100 microcuries per kg.

The present paper describes experiments designed to measure the effect of oxophenarsine (meta-amino-para-hydroxy-phenyl-arsenoxide) on the assimilation of phosphate by *T. equiperdum*.

## MATERIALS AND METHODS

Trypanosome counts were made with a hemocytometer and a red cell diluting pipette, using 0.9% sodium chloride as the diluent.

The radiophosphorus used in this investigation was supplied by Oak Ridge National Laboratory, on authorization from the Isotopes Division, U. S. Atomic Energy Commission.

$P^{32}O_4$ was injected in a slightly acid solution in 0.9% sodium chloride solution and at a concentration of 100 microcuries per ml. The $P^{32}O_4$ solution contained a physiologically negligible amount of phosphorus, i.e., less than 0.25 mg per kg was injected. The injection was made into an exposed femoral vien under ether anesthesia of about 5 minutes duration.

$P^{32}$ counts were made with a thin-walled glass Geiger-Müller tube. Liquid samples of 2 ml were placed in shallow aluminum cups in a fixed position under the counting tube.

Phosphorus was determined by the method of Fiske and Subbarow, with the modification suggested by Gomori.[2]

The trypanosomes were obtained by centrifuging citrated blood. Only the top half of the trypanosome layer was taken, because this affords suspensions practically free of leukocytes. The trypanosomes were washed twice with cold saline and a small amount of material in the tip of the centrifuge tube was discarded after each washing. One ml of each suspension was added to 5 ml of 15% cold trichloroacetic acid as the first step of the fractionation procedure.[3] The precipitates were extracted in turn with: (1) a mixture of 3 parts alcohol and 1 part ether, (2) hot trichloroacetic acid, and (3) finally with a 0.2 *N* sodium hydroxide, which dissolved the rest. This gives 4 phosphorus-bearing fractions, referred to in the literature as acid-soluble phosphorus, phospholipid phosphorus, nucleic acid phosphorus and phosphoprotein phosphorus.

The rats were males weighing around 300 g. In all except the last 2 experiments they were infected by intraperitoneal injection of infected, defibrinated blood 2 to 4 days before they were used. In the last 2 experiments the rats were infected by intravenous injection of defibrinated blood about 18 hours before they were used.

In each experiment there were two infected rats, one of which was treated with oxophenarsine given intravenously and both given $P^{32}$ intravenously. After 2 hours the rats were anesthetized with ether and bled. About 10 ml of blood were usually obtained. The 2-hour interval was chosen on the basis of the results of Moraczewski and Kelsey, who determined the specific activity of the 4 fractions at intervals up to 9 hours. Their results indicate that the specific activity of all the fractions continues to increase for at least 2 hours after injection of $P^{32}O_4$.

Rats having infections of around 1 million trypanosomes per cu mm of blood were used in all but the last two experiments. An attempt was

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1. Moraczewski, S. A. and Kelsey, F. E. 1948, J. Infect. Dis. **82**: 45–51.
2. Gomori, G. 1942, J. Lab. & Clin. Med. **27**: 955–960.
3. Schneider, W. C. 1945, J. Biol. Chem. **161**: 293–303.

TABLE 1.—*The percentage distribution of radioactive phosphate in 4 fractions of treated and untreated trypanosomes in 7 experiments.*

| Exp. | Acid-soluble phosphorus | | Phospholipid phosphorus | | Nucleic acid phosphorus | | Phosphoprotein phosphorus | |
|---|---|---|---|---|---|---|---|---|
| | Treated | Untreated | Treated | Untreated | Treated | Untreated | Treated | Untreated |
| A | 58% | 50% | 9.9% | 21.6% | 29.8% | 25.2% | 2.2% | 3.4% |
| B | 57 | 55 | 14.3 | 20.6 | 26.4 | 21.4 | 2.1 | 3.2 |
| C | 54 | 55 | 12.7 | 18.9 | 32.2 | 23.8 | 1.3 | 1.5 |
| D | 66 | 59 | 10.2 | 17.0 | 21.2 | 21.0 | 2.3 | 2.6 |
| E | 60 | 61 | 10.4 | 16.1 | 27.1 | 19.6 | 3.1 | 3.4 |
| F | 67 | 59 | 10.2 | 17.9 | 22.0 | 21.8 | 1.1 | 1.0 |
| G | 62 | 62 | 12.0 | 16.5 | 24.3 | 20.0 | 1.7 | 1.8 |

These values were obtained by dividing the radioactivity of each fraction by the total radioactivity of the trypanosomes.

made to have two rats with equal infections in each experiment but they varied by as much as 20%. The last two experiments were made with rats having: (1) 398,000 and 333,000; (2) 255,000 and 308,000 trypanosomes per cu mm. In the next to last experiment the lower infection was treated, and in the last experiment the higher infection was treated.

A dose of oxophenarsine (2 micromoles per kg) was found that did not greatly reduce the trypanosome count during the first 2 hours after administration but which is sufficient to clear the trypanosomes from the blood during the 18 hours following administration. This dose did not completely rid the rats of trypanosomes, when they were allowed to live, but reduced the number to a level at which they could not easily be demonstrated microscopically. Eventually, however, the trypanosomes reappeared in the blood and caused death of the rat.

## EXPERIMENTAL RESULTS

Table 1 shows the pattern of distribution of $P^{32}$ in treated and untreated trypanosomes. These data demonstrate a consistent reduction in the relative amounts of phosphate incorporated into phospholipid and phosphoprotein. Since these figures were obtained by dividing the radioactivity of each fraction by the total radioactivity of the trypanosomes, they do not make possible a direct comparison of rates of phosphate uptake in the treated and untreated trypanosomes. It is even conceivable that such changes could occur as the result of varying degrees of stimulation of phosphate metabolism in the different fractions under the influence of the drug.

On the other hand, the specific activity ($P^{32}$ counts per minute per microgram of phosphorus) attained at the end of 2 hours should be a valid measure of the rate of phosphate assimilation during the period of the experiment, since the specific activity is a measure of the extent to which the phosphate present in a given fraction immediately before injection of $P^{32}O_4$ is diluted by new phosphate entering the fraction during the experiment. When these experiments were studied from the standpoint of specific activity of the various fractions the results were not so clear-cut.

In 6 out of the 7 experiments the cold trichloroacetic acid-soluble fraction had a lower specific activity in the treated trypanosomes. The difference ranged from +6% to −51%, with a mean of −24%. In all 7 experiments the phospholipid fraction had a lower specific activity in the treated trypanosomes. The differences varied from −21% to −60%, with a mean of −41%. In 3 of the 7 experiments the nucleic acid fraction of the treated trypanosomes was of a lower specific activity. The differences varied from −36% to +21%, with a mean of +2.4%.

In 5 out of 6 experiments the phosphoprotein fraction of the treated trypanosomes was lower in specific activity than the same fraction of the untreated trypanosomes. The differences varied from −61% to +14%, with a mean of −48%.

The variability of these results sug-

TABLE 2.—*Specific activities* of the phosphate found in four chemical fractions of* T. equiperdum *suspension.*

| | Treated | | | | Untreated | | |
|---|---|---|---|---|---|---|---|
| | Sample 1 | Sample 2 | Mean | Ratio† | Sample 1 | Sample 2 | Mean |
| | | | Experiment 1 | | | | |
| ASP | 33.2 | 34.6 | 33.9 | (0.89) | 39.9 | 36.1 | 38.0 |
| PLP | 13.6 | 12.8 | 13.2 | (0.65) | 21.2 | 19.4 | 20.3 |
| NAP | 12.0 | 11.8 | 11.9 | (0.85) | 14.3 | 13.7 | 14.0 |
| PPP | 5.9 | 4.9 | 5.4 | (0.60) | 8.1 | 9.9 | 9.0 |
| | | | Experiment 2 | | | | |
| ASP | 36.8 | 34.2 | 35.5 | (0.84) | 42.2 | 42.7 | 42.4 |
| PLP | 11.1 | 12.6 | 11.8 | (0.55) | 21.4 | 21.5 | 21.5 |
| NAP | 11.1 | 11.8 | 11.4 | (0.74) | 15.4 | 15.5 | 15.5 |
| PPP | 5.7 | 6.5 | 6.1 | (0.63) | 11.7 | 7.7 | 9.7 |

ASP denotes cold trichloroacetic acid extractable $P^{32}$.
PLP denotes alcohol-ether extractable $P^{32}$.
NAP denotes hot trichloroacetic acid extractable $P^{32}$.
PPP denotes the rest or phosphoprotein $P^{32}$.
* Counts per minute/micrograms of phosphorus.
† Ratio treated mean/untreated mean.

gested that some unrecognized variable existed in the experiments. It seemed likely that the metabolic activity of the trypanosomes might decline at very high levels of infection, such as those used in the experiments. This possibility was tested and it was found that phosphate assimilation does decrease at high levels of infection.[4] Furthermore, Morrell, Chapman and Allmark[5] observed that the rate of increase in parasitemia falls away from the exponential course during the last day of the infection, when the infection rises above 400,000 trypanosomes per cu mm of blood, and Chen, Geiling and McHatton[6] reported the same kind of curve in the mouse. It therefore seemed desirable to make the experiments at lower levels of infection, where the metabolic rate of the trypanosomes may reasonably be expected to be less influenced by small changes in the intensity of the infection.

Soon after the effect of the intensity of the infection on phosphate assimilation was observed it was found that this effect could be greatly reduced by shortening the duration of the infections. When the infections were produced by massive intravenous injection of infected blood about 18 hours before the rats were used, the intensity of the infections became much less important in determining rate of phosphate assimilation.

Experiments were made using both these new observations. In the first experiment two rats with infections of 398,000 trypanosomes per cu mm and 333,000 per cu mm were used. The latter received 2 micromoles per kg of oxophenarsine intravenously, followed immediately by 200 microcuries per kg of $P^{32}O_4$ intravenously. The former received an equivalent volume of normal saline and 200 microcuries of $P^{32}O_4$. After 2 hours the rats were bled and trypanosomes were obtained and subjected to the fractionation procedure. The fractionations were made in duplicate and the phosphate analyses in duplicate, giving duplicate counts of radioactivity and quadruplicate determinations of phosphate by the chemical method.

In table 2 are found the specific activities observed in this experiment and in a similar experiment. In both experiments the phospholipid specific activity and the phosphoprotein specific activity are considerably depressed in the

4. Cantrell, W. 1953. J. Infect. Dis. **93**: 219–221.,
5. Morrell, C. A. Chapman, C. W. and Allmark M. G. 1936. Am. J. Hyg. **25**: 232–258.
6. Chen, G., Geiling, E. M. K. and McHatton, R. M. 1945. Am. J. Hyg. **41**: 221–227.

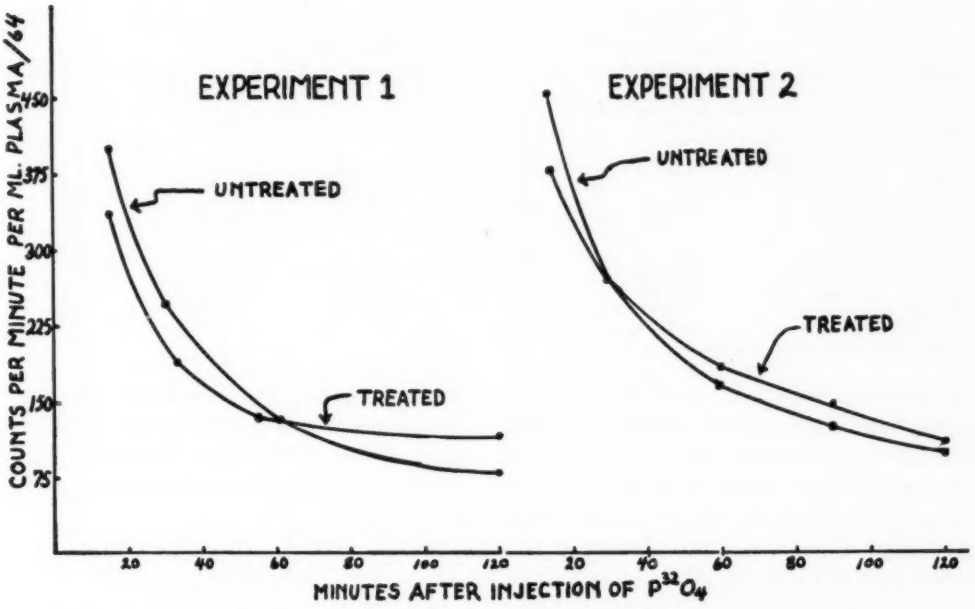


FIG. 1.—Radioactivity of plasma of 4 rats after injection of 200 microcuries per kg of $P^{32}O_4$.

treated trypanosomes, while the acid-soluble specific activity is least affected and the nucleic acid specific activity is depressed an intermediate amount.

In table 3 the distribution of phosphate among the 4 fractions is shown. These figures are the results of ordinary chemical analysis by the method of Fiske and Subbarow. The values in the untreated columns of experiment 1 are low because of some difficulties encountered in obtaining the trypanosomes from the infected blood. In experiment 2 more nearly equal samples of the trypanosomes were obtained, and the specific activities are quite similar to those of experiment 1. In both experiments the phospholipid phosphorus in the treated trypanosomes makes up a smaller percentage of the total phosphorus and the acid-soluble phosphorus makes up a larger percentage of the total in the treated trypanosomes.

Table 4 shows the distribution of radioactivity among the 4 fractions and is included mainly to record the magnitude of the errors in determining the radioactivity in these experiments. It

TABLE 3.—*Distribution of phosphate among 4 chemical fractions of* T. equiperdum *suspensions.*

| | Treated | | Untreated | |
|---|---|---|---|---|
| | Sample 1 | Sample 2 | Sample 1 | Sample 2 |
| | | Experiment 1 | | |
| ASP | 55.6 μg (50%) | 57.9 μg (49%) | 10.9 μg (39%) | 11.2 μg (38%) |
| PLP | 18.0 μg (16%) | 18.6 μg (16%) | 6.2 μg (22%) | 6.9 μg (24%) |
| NAP | 32.8 μg (29%) | 34.8 μg (30%) | 8.8 μg (31%) | 8.8 μg (30%) |
| PPP | 4.9 μg ( 4%) | 5.9 μg ( 5%) | 2.4 μg ( 8%) | 2.4 μg ( 8%) |
| | | Experiment 2 | | |
| ASP | 38.2 μg (45%) | 38.4 μg (46)% | 47.8 μg (38%) | 48.7 μg (39%) |
| PLP | 14.5 μg (17%) | 13.9 μg (17%) | 25.8 μg (21%) | 25.7 μg (20%) |
| NAP | 26.8 μg (32%) | 26.0 μg (31%) | 45.0 μg (36%) | 44.8 μg (35%) |
| PPP | 5.4 μg ( 6%) | 5.8 μg ( 7%) | 6.8 μg ( 5%) | 7.1 μg ( 6%) |

ASP, PLP, NAP, PPP—see table 2.

TABLE 4.—*$P^{32}$ taken up by treated and untreated trypanosomes. Counts per $10^9$ trypanosomes based on counts per 189μg of phosphate.*

| | Treated | | | Untreated | | |
|---|---|---|---|---|---|---|
| | Sample 1 | Sample 2 | Mean | Sample 1 | Sample 2 | Mean |
| | | | Experiment 1 | | | |
| ASP | 3145 ± 42 | 3000 ± 40 | 3072 | 3030 ± 93 | 2530 ± 83 | 2780 |
| PLP | 440 ± 17 | 384 ± 16 | 412 | 881 ± 40 | 865 ± 39 | 873 |
| NAP | 675 ± 15 | 662 ± 15 | 668 | 810 ± 39 | 782 ± 40 | 796 |
| PPP | 49 ± 8 | 53 ± 10 | 51 | 127 ± 33 | 161 ± 32 | 144 |
| | | | Experiment 2 | | | |
| ASP | 3140 ± 51 | 2970 ± 42 | 3055 | 3060 ± 39 | 3120 ± 39 | 3090 |
| PLP | 361 ± 20 | 385 ± 20 | 373 | 830 ± 20 | 826 ± 19 | 828 |
| NAP | 666 ± 27 | 690 ± 27 | 678 | 1040 ± 24 | 1040 ± 24 | 1040 |
| PPP | 69 ± 11 | 85 ± 11 | 77 | 121 ± 17 | 82 ± 12 | 101 |

ASP, PLP, NAP, PPP—see table 2.

should be pointed out that these results have been expressed in terms of counts per $10^9$ trypanosomes by using the total phosphate as a measure of the number of trypanosomes in the samples. This was done because of the difficulty encountered in counting the suspensions of treated trypanosomes, which do not distribute themselves evenly in the hemocytometer but form clumps of trypanosomes which make exact counting impossible.

The concentration of $P^{32}$ in the plasma was measured at intervals during the experiments and the curves shown in figure 1 were obtained. The areas under the curves were measured with a planimeter, and in experiment 1 the areas were within 1% of each other and in experiment 2 within 4% of each other. No attempt has been made to extrapolate these curves back to the injection time. These results indicate that the trypanosomes in the treated and untreated rats were subjected to a similar environment with respect to $P^{32}$.

## DISCUSSION

These experiments seem to the author to be evidence against the hypothesis that oxophenarsine kills *T. equiperdum* by interfering with glucose metabolism, since the intermediates of glucose metabolism occur in the fraction least affected—the cold trichloroacetic acid fraction.[7,8] They point to phospholipid metabolism or phosphoprotein metabolism as more likely sites of the primary effect of the drug since these fractions were most affected by the presence of the drug.

## SUMMARY

The phosphate assimilation of *Trypanosoma equiperdum* in treated and untreated rats was compared with the aid of radioactive phosphate. Changes in the pattern of phosphate assimilation were found when the radioactivity of each of four fractions was expressed as a percentage of the total radioactivity of the trypanosomes. Under the influence of oxophenarsine a smaller percentage of the radioactive phosphate of the trypanosomes was present in the phospholipid and phosphoprotein fractions and a larger percentage was incorporated in nucleic acid.

Using specific activity as an indicator of the rate of phosphate assimilation, it was found that oxophenarsine depressed phosphate assimilation in all four fractions studied, the phospholipid and phosphoprotein fractions being most affected and the cold trichloroacetic acid soluble fraction least affected.

Considering all the phosphate, both

7. Chen, G., and Geiling, E. M. K. 1946, Proc. Soc. Exper. Biol. & Med. **63**: 486.
8. Harvey, S. C. 1948, J. Biol. Chem. **179**: 435–453.

radioactive and nonradioactive, in the trypanosomes, a smaller percentage of the total phosphate was present in the phospholipid fraction of the treated trypanosomes.

To avoid variations in the rate of phosphate metabolism, unrelated to the presence of the drug, experiments were made with infections of short duration (18 hours) and relatively low parasite density (less than 400,000 trypanosomes per cubic mm).

# HISTOPLASMOSIS: PATHOLOGIC STUDIES OF FIFTY CATS AND FIFTY DOGS FROM LOUDOUN COUNTY, VIRGINIA

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Previous investigations have demonstrated a high incidence of isolation of *Histoplasma capsulatum* from cats and dogs obtained in Loudoun County, Virginia.[1,2] A rabies control program in that area in the summer of 1952 afforded an unusual opportunity to obtain large numbers of presumably normal cats and dogs. An intensive study of histoplasmosis in the naturally infected cat and dog was thereby possible. The investigation was designed to include skin testing, serology, and gross and histopathological studies, as well as cultures of selected tissues, for fungi on each animal.

## METHODS AND MATERIAL

*Animals.*—The group for this study consisted of 50 consecutive adult cats and 50 consecutive adult dogs brought to the laboratory from this county for any reason. Three of these cats were brought to the laboratory because of suspected rabies. Rabies was demonstrated in only one, a second cat had severe bilateral ocular injuries, while the third cat showed no gross pathology. Four other cats were brought in with obvious lesions: one had a large supraorbital tumor, one had an injury of one ear and a healed amputation of the right hind lower leg, one had a healed amputation of the right fore leg, and one had a draining sinus at the base of the tail. The remaining 43 cats were apparently healthy, unwanted adult animals obtained from the owners.

One dog was brought in because of suspected rabies which was confirmed. The remaining 49 dogs were either stray dogs or dogs belonging to owners who preferred to dispose of them. The animals were obtained from premises widely distributed over Loudoun County, which has an area of about 517 square miles (fig. 1).

*Skin testing.*—One-tenth ml of a 1:100 dilution of histoplasmin was injected intradermally on a shaved area high on the neck of the dogs and on the flanks of the cats. The test was read at 24 hours. An area of induration 0.5 cm or greater in diameter was considered a positive reaction.

*Cultures.*—Cultures on each animal included: superficial anterior cervical lymph nodes (2 to 4 cultures), deep anterior cervical lymph nodes (2 to 4 cultures), submaxillary glands (2 cultures), peribronchial lymph nodes (2 to 6 cultures), liver (2 cultures), spleen (2 cultures), mesenteric lymph nodes (1 to 4 cultures), and adrenal (1 culture). Additional cultures were obtained when possible from the supraclavicular, axillary, anterior mediastinal, and inguinal lymph nodes and from any gross lesion. Fifteen to 25 cultures were usually obtained on each cat and 20 to 30 cultures on each dog. All tissues were cultured on slants of modified Sabouraud's glucose agar.*

A separate set of sterile instruments was used for obtaining samples of each tissue cultured. Samples approximately 5×5×5 mm were placed on the slant; the tissues were macerated by several cuts with the scissors used to obtain the sample. The fragments were streaked over the slant with a sterile inoculating needle and the fragments were left scattered over the surface of the slant. Cultures were incubated at 30 C and were examined at weekly intervals for a period of 6 weeks. All suspicious cultures were examined microscopically and *H. capsulatum* was identified by the characteristic microscopic morphology.

*Gross pathology and histopathology.*—After obtaining cultures, a complete autopsy was done, excluding the brain, except in suspected rabies-

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* Laboratory of Infectious Diseases, National Microbiological Institute.

† Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases.

1. Emmons, C. W. 1949, Histoplasmosis in animals. Tr. N. Y. Acad. Sci., Ser. 2, **2**: 248–254.
2. Emmons, C. W. 1950, Histoplasmosis: animal reservoirs and other sources in nature of the pathogenic fungus, *Histoplasma*. Am. J. Pub. Health. **40**: 436–440.

* 1% neopeptone (Difco), 2% glucose, and 2% agar.

infected animals. Specimens for section were obtained from all tissues cultured for fungi as well as from heart, lungs, kidneys, gastrointestinal tract, and certain endocrine glands. Tissues were fixed in 10% formalin. Four stains, hematoxylin and eosin, azure and eosin, trichrome, and Bauer's, were done on sections of each tissue.

*Serology.*—After the animal became unconscious from the subcutaneous injection of a sterile anesthetic agent 10 ml of blood were obtained by heart puncture using sterile technique. All serums were transferred from the blood clot within 24 hours and were stored in stoppered sealed tubes at −30 C. Titrations for antibody were done at the conclusion of the study, using the same agglutinin or complement-fixation system for all serums.

For collodion particle agglutinins the particles were prepared after the method of Cannon and Marshall.[3] Particles were sensitized by adding 60 ml of particle suspension to an equal volume of undiluted histoplasmin and incubating the suspension at room temperature for 2 hours. The sensitized particles were washed one time and resuspended in 240 ml of distilled water; this suspension had a turbidity corresponding to tube 5 of the McFarland scale. One-half ml of the sensitized particle suspension was added to an equal volume of serum dilution, and the titrations were then carried out as described by the above investigators.

For the complement-fixation test heat-killed whole yeast cells of *H. capsulatum* "strain" 6617 were used as antigen. The yeast cells were harvested from 2-day-old cultures grown on glucose cystine blood agar.* The yeast cells were washed three times, each time in approximately 800 ml of saline. After the last washing 10 ml of packed cells were resuspended in 90 ml of saline. The suspension was then heat-killed at 57 C for 90 minutes. The suspension was stored at 4 C and was used for antibody titrations within a week after preparation. This suspension had no anticomplementary activity. The complement-fixation test was that described by Bengtson.[4] Two full units of complement, 2 units of hemolysin, and 4 units of antigen were used. Antigen was titrated against serums from 5 dogs naturally infected with histoplasma and against serums from laboratory-infected rabbits. A dilution of packed cells to saline of 1:50 was titrated as 4 units of antigen. The titer was considered as the highest serum dilution in which no visible hemolysis occurred.

For both agglutinins and complement-fixation titrations serums were inactivated at 60 C for 20 minutes. The initial serum dilution for the complement-fixation titration was 1:15, the dilution of serum before the addition of the hemolytic system. The initial serum dilution for the agglutinins was 1:20, the dilution of serum after the addition of collodion particles. All serums were titrated for anticomplementary activity and all serums were tested for agglutinins for nonsensitized collodion particles.

## RESULTS

*Cats.*—*H. capsulatum* was isolated from 22 of the 50 cats. The organism was isolated from 18 of 37 females and from 4 of 13 males. In 8 cats the only isolation of *H. capsulatum* was from the peribronchial lymph nodes, and in an additional 9 cats the organism was isolated from the peribronchial lymph nodes as well as from other sites. In 2 cats the only isolation was from the cervical lymph nodes. In no cat was *H. capsulatum* isolated solely from the abdominal organs, i.e., from liver, spleen, mesenteric lymph nodes, or adrenal. Dissemination of the organism, as indicated by multiple positive cultures from the abdominal organs as well as from the cervical and/or peribronchial lymph nodes, was present in 8 cats.

Only one cat had a positive skin test and *H. capsulatum* was isolated from the cervical and peribronchial lymph nodes of this animal. None of the cat serums showed complement-fixing antibodies to the whole yeast phase antigen; none of

3. Cannon, P. R. and Marshall, C. E. 1940, An improved serologic method for the determination of the precipitative titers of antisera. J. Immunol. **38:** 365–376.

* For 1 liter of medium 1.0 g of cystine was dissolved in 1 liter of melted base at 60 C for 12 hours. The base contained 5% beef heart infusion (Difco), 1% proteose peptone (Difco), 0.5% NaCl, and 1.5% agar. The solution was cooled to 50 C, and 20 ml of 50% sterile glucose and 60 ml of fresh whole defibrinated rabbit blood were added. The medium was immediately dispensed, 75 ml of medium per 1 liter Blake flask.

4. Bengtson, I. A. 1944, Complement-fixation in Rickettsial diseases—technique of the test. Pub. Health Rep. **59:** 402–405.

FIG. 1.—Loudoun County, Virginia. Points where 50 cats and 50 dogs were obtained.

the cat serums were anticomplementary. Six of the cat serums had agglutinins for nonsensitized collodion particles; the remaining 44 cat serums showed no agglutinins for sensitized collodion particles.

No characteristic gross or histopathologic lesion was associated with isolation of *H. capsulatum* from cat tissues. No intracellular yeast cells were seen in hematoxylin and eosin- or Bauer-stained histologic sections from any of the cats.

It is of interest that *H. capsulatum* was isolated from 5 of the 6 cats brought in with gross external abnormalities, though there is no apparent association between the cause of the abnormalities and infection with histoplasma. *H. capsulatum* was isolated from the peribronchial lymph nodes of the cat infected with rabies, from the cervical lymph nodes and spleen of the cat with bilateral ocular injuries, and from the peribronchial lymph nodes and spleen of the cat with a supraorbital neurofibrosarcoma. The 2 cats with the largest number of positive cultures for *H. capsulatum* and with evidence of the widest

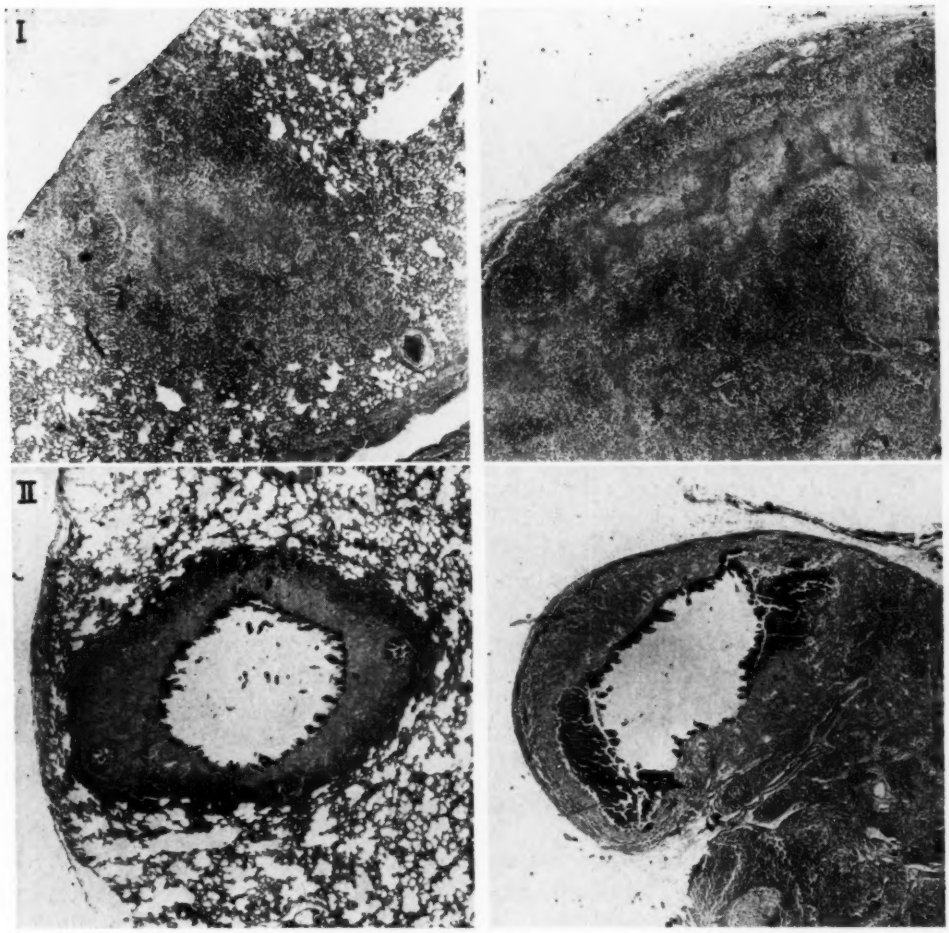


PLATE I.—Photomicrographs of hematoxylin and eosin-stained sections from dog 28. Granulomatous type lesions with areas of central caseous necrosis. Left, lung, ×17; right, peribronchial lymph node, ×17.

PLATE II.—Photomicrographs of hematoxylin and eosin-stained sections from dog 14. Organized granulomatous type lesions with central deposits of calcium. Left, lung, ×17; Right, peribronchial lymph node, ×17.

dissemination of the organism both had healed amputations. Only 2 cats were in apparent poor nutritional status; *H. capsulatum* was isolated from the cervical lymph nodes from one of these cats.

Of the 37 female cats 6 were pregnant and 11 were either brought in with suckling kittens or showed evidence of recent parturition. *H. capsulatum* was isolated from 4 of the 6 pregnant cats and from 5 of the 11 cats which had recently delivered kittens.

In 5 cats small pleural scars were found. The lesions were 1 to 3 mm in diameter, slightly raised, soft, and tan to yellow in color; these scars were sometimes associated with soft discrete yellow nodules, 1 to 2 mm in diameter, within the lung parenchyma and/or in the peribronchial lymph nodes. In these 5 cats and in 1 cat with no gross lesions, a verminous pneumonia was found. The pneumonia in all cases was caused by nematode larvae with morphologic char-

acteristics of *Aeleurostrongulus abstrusus*. *H. capsulatum* was isolated from 2 of the 6 cats with verminous pneumonia; the only isolation from each of these 2 cats was from the peribronchial lymph nodes.

Sections of lung from 1 cat showed a large encapsulated nematode larva which could not be further identified; *H. capsulatum* was isolated from the peribronchial lymph nodes and spleen of this cat. There was parasitism of tracheal and bronchial mucosa with eggs of *Capillaria aerophilia* in 2 cats; *H. capsulatum* was isolated from the cervical lymph nodes from one of these cats. There was parasitism of the cervical musculature with *Sarcocystis* in 2 cats; *H. capsulatum* was isolated from the spleen, peribronchial lymph nodes, and mesenteric lymph nodes from one of these cats. A variety of intestinal parasites was present in a large number of the cats, but there was no apparent relationship between the isolation of *H. capsulatum* and the presence of these parasites in the cats.

A chronic cholecystitis with no associated liver or pancreas pathology was found in 5 cats; *H. capsulatum* was isolated from the peribronchial and cervical lymph nodes from 1 of these 5 cats.

*Dogs.*—*H. capsulatum* was isolated from 22 of the 50 dogs. The organism was isolated from 14 of 25 females and from 8 of 25 males. In 3 dogs the only isolation of *H. capsulatum* was from the peribronchial lymph nodes, and in an additional 9 dogs the organism was isolated from the peribronchial lymph nodes as well as from other sites. In 6 dogs the only isolation was from the cervical lymph nodes. In 2 dogs *H. capsulatum* was isolated solely from the abdominal organs, from the liver in 1 dog and from the spleen and mesenteric lymph nodes of the other. Dissemination of the organism as indicated by multiple positive cultures from the abdominal organs as well as from the cervical and/or peribronchial lymph nodes was present in 9 dogs.

Forty-nine of the 50 dogs were skin-tested; 14 had positive reactions to histoplasmin. Twenty-seven dogs had agglutinin titers of 1:20 or higher and 15 dogs had complement-fixation titers of 1:15 or higher. The data on isolation of the organism by culture as compared with skin test reactions and serological reactions are presented in tables 1 and 2. Two dog serums had nonspecific agglutinins for unsensitized collodion particles and 2 other serums were anticomplementary. In none of these 4 dogs was *H. capsulatum* isolated nor was the skin test or other serologic test positive. These four dogs are not included in table 1 and are represented in the first column in table 2.

Only 2 of the 50 dogs were obviously ill on arrival at the laboratory. Dog 4, which was rabid, was semiconscious and markedly dehydrated. *H. capsulatum* was isolated from the peribronchial lymph nodes of this dog. Dog 21 was in good nutritional status but had respiratory difficulty and stridor on exertion. This dog had a large, 10×10×10 cm, hard tumor mass which surrounded the distal trachea and main bronchi and had invaded the hilum of each lung lobe. There was invasion of the trachea, with constriction of the lumen to less than 0.5 cm in diameter. The tumor proved to be a plasma cell sarcoma. *H. capsulatum* was isolated from the cervical lymph nodes of this dog.

Two aged dogs, 22 and 23, were edentulous, deaf, and blind. Both had myocardial fibrosis, cystitis, and pyelonephritis. One had an interstitial cell carcinoma of the testis and the other had an adenoma of the adrenal. A third dog had a mediastinal teratoma and an old empyema with extensive pleural ad-

TABLE 1.—*Summary of laboratory examinations of 33 dogs.**

| Dog number | Skin test | Serology | | Cultures | | Pathology | |
|---|---|---|---|---|---|---|---|
| | Mm induration | Agglutination titer | Complement-fixation titer | Number of positive cultures for histoplasma | Tissues histoplasma isolated from: | Lung and peribronchial lymph node lesions | Tissue histoplasma demonstrated in: |
| 1 | 0 | 0 | 30 | 0 | 0 | Lu, PLN | 0 |
| 2 | 0 | 0 | 0 | 1 | Liv | Lu | Liv |
| 3 | 10×10 | 40 | 0 | 4 | Spl, MLN | 0 | Spl, MLN |
| 4 | N.D. | 0 | 0 | 1 | PLN | 0 | 0 |
| 5 | 0 | 80 | 0 | 3 | PLN | Lu, PLN | PLN |
| 6 | 13×15 | 160 | 30 | 6 | PLN, Spl | Lu | 0 |
| 7 | 15×20 | 80 | 0 | 1 | CLN | 0 | 0 |
| 8 | 0 | 20 | 0 | 0 | 0 | Lu, PLN | 0 |
| 9 | 0 | 0 | 15 | 6 | Liv, Spl, MLN | 0 | 0 |
| 10 | 0 | 0 | 0 | 2 | PLN, CLN | Lu | PLN |
| 11 | 10×10 | 160 | 60 | 0 | 0 | PLN | 0 |
| 12 | 5×5 | 80 | 0 | 3 | PLN, CLN, MLN | PLN | PLN |
| 13 | 0 | 20 | 0 | 0 | 0 | Lu | 0 |
| 14 | 10×10 | 0 | 0 | 2 | PLN | Lu, PLN | 0 |
| 15 | 10×10 | 160 | 60 | 3 | CLN | Lu, PLN | 0 |
| 16 | 0 | 0 | 60 | 4 | CLN, PLN, MLN | 0 | 0 |
| 17 | 0 | 160 | 0 | 4 | MeLN, Liv | 0 | 0 |
| 18 | 0 | 0 | 60 | 25 | CLN, PLN, Liv, Spl, MLN, Adr, Om, Mes | Lu, PLN | PLN, Om, Mes |
| 19 | 5×5 | 0 | 0 | 1 | CLN | Lu | 0 |
| 20 | 0 | 160 | 30 | 1 | CLN | Lu | 0 |
| 21 | 10×15 | 0 | 30 | 2 | CLN | Lu | 0 |
| 22 | 0 | 20 | 60 | 0 | 0 | 0 | 0 |
| 23 | 0 | 40 | 30 | 0 | 0 | 0 | 0 |
| 24 | 20×20 | 160 | 0 | 1 | CLN | Lu | 0 |
| 25 | 0 | 0 | 30 | 2 | CLN | Lu | 0 |
| 26 | 0 | 80 | 0 | 5 | PLN, Spl | Lu, PLN | PLN, Liv |
| 27 | 5×5 | 160 | 30 | 0 | 0 | 0 | 0 |
| 28 | 5×5 | 80 | 15 | 6 | PLN, MLN, Spl | Lu, PLN | 0 |
| 29 | 0 | 160 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 320 | 0 | 0 | 0 | PLN | 0 |
| 31 | 0 | 160 | 0 | 0 | 0 | 0 | 0 |
| 32 | 10×10 | 160 | 0 | 3 | PLN, MLN | PLN | PLN |
| 33 | 20×20 | 80 | 15 | 0 | 0 | 0 | 0 |

N.D. =not done, Liv = liver, Spl =spleen, Lu =lung, CLN =cervical lymph node, PLN =peribronchial lymph node MeLN =anteriol mediastinal lymph node, MLN =mesenteric lymph node, Adr =adrenal, Om =omentum, Mes =mesentery
* 5 dogs not included had agglutinin titers of: 20, 40, 80, 80 and 80. 12 dogs not included had no positive cultures, skin tests or serologic tests.

hesions. *H. capsulatum* was not isolated from these three dogs.

Of the 25 female dogs 3 were pregnant and 3 were either brought in with suckling puppies or showed evidence of recent parturition. *H. capsulatum* was isolated from 2 of the 3 pregnant bitches and from 1 of the 3 recently pregnant bitches.

At autopsy discrete intrapulmonary nodules were found in 13 dogs. There were single nodules in 4 dogs and multiple nodules in 9 dogs. The lesions varied from approximately 0.5 to 3.0 mm in diameter. They were tan to yellow in color; some were soft and caseous, while others were firm and apparently calcified. In 3 dogs both types of nodules were found. In 5 of these 13 dogs small, 0.5 to 1.0 mm, yellow nodules were also found in the peribronchial lymph nodes. Histologic examination of these intrapulmonary and lymph node nodules showed a variety of lesions. Some were circumscribed areas consisting primarily of large mononuclear cells and a

TABLE 2.—*Tabulation of 49 dogs according to results of laboratory examinations.**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cultures | 0 | 0 | 0 | 0 | 0 | + | + | + | + | + | + | + | + |
| Skin test | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | + | + | + | + |
| Serology Agglutination | 0 | 0 | + | + | + | 0 | 0 | + | + | 0 | 0 | + | + |
| Serology Complement-fixation | 0 | + | 0 | + | + | 0 | + | 0 | + | 0 | + | 0 | + |
| Numbers of dogs with above combination of attributes | 12 | 1 | 10 | 2 | 3 | 2 | 4 | 3 | 1 | 2 | 1 | 5 | 3 |

Agglutinin titer of 1:20 or higher considered as positive.
Complement-fixation titer of 1:15 or higher considered as positive.
* One dog was not skin tested; *H. capsulatum* was isolated from this animal. The titers for agglutinins and complement-fixation were negative.

few polymorphonuclear leukocytes, lymphocytes, and plasma cells. Some were circumscribed granulomatous type lesions, with central caseous necrosis, while others were highly organized encapsulated lesions with central deposits of calcium (plate I and II). Similar types of small lesions were found in histologic sections from 8 dogs in which no gross pathology was noted. No animal parasites were found associated with pulmonary lesions in any of the dogs.

All told, these pulmonary and/or peribronchial lymph node lesions were found in 21 dogs and *H. capsulatum* was isolated from 16 of these dogs. Direct cultures of gross intrapulmonary nodules were obtained from 5 dogs; cultures from 2 were sterile, cultures from 3 were overgrown by a variety of saprophytic fungi. *H. capsulatum* was isolated from the peribronchial lymph nodes from 9 dogs and from other tissues, either the cervical lymph nodes or liver, from 7 of these 21 dogs. All cultures of the peribronchial lymph nodes of these 7 dogs and of the 5 dogs from which no *H. capsulatum* was isolated were overgrown by saprophytic fungi. *H. capsulatum* was isolated from peribronchial lymph nodes with either small focal areas of cellular infiltrates or from those with the more highly organized lesions with central caseous necrosis or with central deposits of calcium as well as from nodes which had no lesions. *H. capsulatum* was isolated from 6 dogs which had no pulmonary or peribronchial lymph node lesions.

*H. capsulatum* was found histologically in sections from 8 of the 50 dogs and was isolated in culture from these 8 dogs in all but one instance from the tissue in which the yeast cells were seen microscopically. In sections from only one dog, 18, is *H. capsulatum* found readily. In the hematoxylin and eosin stained sections the yeast cells were very difficult to find. However, in the Bauer-stained sections from this dog the yeast cells were found more readily and in greater numbers though rarely were more than 2 cells seen within a single large mononuclear cell. No *H. capsulatum* was demonstrated in any of the hematoxylin and eosin-stained sections of the other 49 dogs. In Bauer-stained sections from 7 dogs a very few, 1 to 5, intracellular yeast cells were found. In most instances they were present in a large mononuclear cell lying free in a lymph node sinusoid. *H. capsulatum* has not been demonstrated histologically in or on the periphery of any of the lung or peribronchial lymph node lesions.

## DISCUSSION

The results of this study substantiate and extend the observation that naturally acquired mild histoplasmosis is a widely prevalent infection among cats and dogs in Loudoun County, Virginia. *H. capsulatum* was isolated most frequently from the cervical and peribronchial lymph nodes of both cats and dogs, and this may indicate that infection occurs frequently by inhalation of spores of the saprophytic phase of the fungus. The fact that *H. capsulatum* has been isolated frequently from soil obtained from premises widely scattered over Loudon County gives support to this suggestion.[5]

Whether or not the infections we detect are recent infections or reinfections cannot be stated, though evidence of dissemination of the organism was present in 17 of the 44 infected animals. In the past 7 years we know of only 5 dogs from Loudoun County having disseminated histoplasmosis with emaciation, hepatomegaly, splenomegaly, as-

5. Emmons, C. W. 1949, Isolation of *Histoplasma capsulatum* from soil. Pub. Health Rep. **64**: 892–896.

cites, and melena. No investigations on the ability of cats to clear experimentally induced infection with *H. capsulatum* have been reported and no adequate studies have been reported on dogs. It is of interest that *H. capsulatum* was isolated from guinea pigs 2 years after the induction of experimental infection, and those animals showed no clinical illness during that interval.[6] *H. capsulatum* has been isolated from 4 kittens 4 months after the experimental induction of infection. During this 4-month interval the kittens gained weight normally and no gross or histopathologic lesions were present in these kittens. Similarly, *H. capsulatum* has been uniformly isolated from mice, rats, rabbits, and guinea pigs 6 months after the induction of infection with fewer than 100 yeast cells of *H. capsulatum*.[7]

The results of this study indicate that dogs react immunologically at least quantitatively differently than cats. The four kittens experimentally infected did react strongly to the skin test as performed in these studies, though, like the naturally infected cats, showed no agglutinins or complement-fixing antibodies to histoplasma.

Neither skin nor serologic tests as performed in this study gave a satisfactory index of whether or not *H. capsulatum* could be isolated from the dog. The organism was isolated from 10 dogs with negative skin tests, from 9 dogs with negative agglutinin titers and from 12 dogs with negative complement-fixation titers. Also there was failure to isolate the organism from 3 dogs which had positive reactions to all 3 immunologic tests.

For skin testing the dilution of antigen chosen was arbitrary. Lot H3 histoplasmin prepared in this laboratory was used; this histoplasmin diluted 1:1000 has been used extensively for human epidemiological studies in Loudoun County. Subsequent to the conclusion of this study standards were set up for the minimum requirements of histoplasmin as a skin test antigen.[8] Our facilities for housing large numbers of cats and dogs were limited, and it was necessary to autopsy the animals within 24 hours after arrival at the laboratory. The skin tests were not read at the optimum time, which is considered to be 48 hours for the dog.[9,10] It is possible that if a different antigen preparation or a different dilution of the H-3 histoplasmin had been used and the skin tests had been read at 48 hours, a better correlation between skin test reactions and isolation of the fungus would have been found.

The serologic tests as performed in these studies are not necessarily comparable to tests done by other workers in this field. For collodion particle agglutinins a different lot of histoplasmin was used and the methods for preparing and sensitizing the particles were different than the methods previously described.[11] For the complement-fixatin tests the whole yeast phase antigen was arbitrarily chosen as the antigen of

6. Emmons, C. W., Bell, J. A. and Olson, B. J. 1947, Naturally occurring histoplasmosis in *mus musculus* and *Rattus norvegicus*. Pub. Health Rep. **62**: 1642–1646.
7. Rowley, D. A. Unpublished data.
8. Minimum requirements: histoplasmin. Oct. 27, 1952, National Institutes of Health, Bethesda 14, Md.
9. Prior, J. A., Cole, C. R. and Torbet, M. S. 1949, An evaluation of the histoplasmin reaction in the detection of naturally occurring histoplasmosis in dogs. Pub. Health Rep. **64**: 1562–1566.
10. Menges, R. W. 1951, The histoplasmin skin test in animals. J. A. V. M. A. **119**: 69–71.
11. Saslaw, S. and Campbell, C. C. 1948, A method for demonstrating antibodies in rabbit sera against histoplasmin by the collodion agglutination technic. Proc. Soc. Exper. Biol. & Med. **68**: 559–562.

choice, though other workers have preferred to use histoplasmin or the filtrate of ground whole yeast phase antigens for this test.[12,13] There are contradictory reports on the sensitivity of complement-fixation tests using the whole yeast phase antigen as compared to the filtrate of ground yeast phase antigen.[13,14] Each investigator who has prepared yeast phase antigens for complement-fixation tests has used different strains of *H. capsulatum*, different mediums for growing the organism, different aged cultures, and different methods for killing and storing the antigen preparations.[13–15] The methods used for preparing the whole yeast phase antigen for this study differ from the methods used by the investigators above. It is possible that if different serologic tests or different antigen preparations had been used a better correlation between serologic reactions and isolation of the fungus would have been found.

There are limitations to the methods used for detecting infection by isolation of the organism. *H. capsulatum* is slow growing and cultures can seldom be positively identified before 10 days after isolation. Most saprophytic fungi grow much more rapidly, and the presence of these fungi obscures or prevents the growth of *H. capsulatum*. Contamination was limited almost exclusively to cultures of the peribronchial lymph nodes, where it was frequently found that 4 or 5 different fungi were isolated from this tissue, though cultures of all other tissues from the same animal were sterile. Nevertheless, *H. capsulatum* was isolated solely from the peribronchial lymph nodes of 11 of the 44 infected animals in this series, yet all cultures of the peribronchial lymph nodes were overgrown by saprophytic fungi from 22 animals with no positive cultures for *H. capsulatum*. Only a fraction of the total possibly infected tissue was cultured and the tissue most likely to yield *H. capsulatum* in culture, viz., peribronchial lymph nodes, frequently contained viable spores of saprophytic fungi.

Studies on the sensitivity of the cultural technique for demonstrating *H. capsulatum* in noncontaminated dog tissues have not been carried out. It is of interest that multiple cultures of *H. capsulatum* have been obtained from livers, spleens, and lungs of mice injected intravenously with fewer than 100 yeast cells of *H. capsulatum* and sacrificed 1 hour after injection.[7] Though Sabouraud's glucose agar does not support good germination of isolated single yeast cells to mycelial colonies,[7] apparently the addition of the tissue fragments to the slant makes it an adequate medium for demonstrating the presence of a very few yeast cells in mouse tissue.

In spite of the limitations of the cultural technique for demonstrating the presence of the organism in infected tissues, this method is certainly more delicate than demonstrating the organism in histologic sections. In sections from fatal disseminated histoplasmosis of either man or dog the organism can be found readily in hematoxylin and eosin-stained material, and there is little need of special stains for polysaccharide such as the Bauer stain. However, in sections

12. Furcolow, M. L., Bunnell, I. L. and Tennenberg, D. J. 1948, A complement-fixation test for histoplasmosis. II. Preliminary results with human sera. Pub. Health Rep. **63**: 169–178.
13. Campbell, C. C., Saslaw, S. and Knibb, S. A. 1948, The use of yeast phase antigens in complement-fixation test for histoplasmosis. II. Results with ground antigens. J. Lab. Clin. Med. **33**: 1207–1211.
14. Grayston, J. T. 1952, A study of the complement-fixation reaction in histoplasmosis. J. Lab. Clin. Med. **40**: 90–101.
15. Salvin, S. B. 1947, Complement-fixation studies in experimental histoplasmosis. Proc. Soc. Exper. Biol. & Med. **66**: 342–345.

from naturally infected animals in this study the organism was found in the hematoxylin and eosin-stained sections from only one dog. With the aid of a polysaccharide stain the organism was found in sections from an additional 7 dogs.

The data are insufficient to evaluate factors such as age, sex, nutritional status, injuries, malignancy, or parasitism which may condition infection with *H. capsulatum* in cats and dogs. Also the data are insufficient to determine whether any of the intrapulmonary and peribronchial lymph node lesions in dogs are caused by *H. capsulatum*.

Studies on cats and dogs obtained from Loudoun County subsequent to this series will be reported in detail at a later date. However, certain aspects of these subsequent investigations are pertinent to the present study. *H. capsulatum* has been isolated from 12 of 34 cats and from 66 of 154 dogs. Cats were skin tested, using 0.1 ml of undiluted Lot H3 histoplasmin; dogs were skin tested as reported in this study. All skin tests were read at 24 and 48 hours. None of the cats had positive skin tests. Thirty-five of the 154 dogs had positive skin tests. Twenty-one had positive tests at 24 and 48 hours and 14 had negative tests at 24 hours but positive tests at 48 hours. *H. capsulatum* was isolated from 43 dogs with negative skin tests at 24 and 48 hours.

Serums were obtained from 40 dogs prior to skin testing and 48 hours after skin testing; skin testing had no apparent affect on either collodion particle agglutinin or complement-fixation titers of these 40 dogs.

Comparative studies on isolation of *H. capsulatum* from peribronchial lymph nodes by direct culture and by mouse passage have been carried out. Peribronchial lymph nodes from each dog were pooled and one-half of the tissue was cultured directly as in this study and the other half was homogenized and injected into mice. The mice were autopsied 4 to 6 weeks after the lymph node injection, and cultures were obtained from the livers and spleens of the mice. By using this technique *H. capsulatum* has been isolated from the peribronchial lymph nodes of many dogs from which the direct cultures of this tissue were overgrown by saprophytic fungi. A variety of intrapulmonary lesions from 12 dogs have also been homogenized and passed through mice. With this technique *H. capsulatum* was isolated from the lesions from one dog, though the significance of this finding is doubtful since positive direct cultures were obtained in 25 cultures from 6 other tissues of this dog.

## SUMMARY

*Histoplasma capsulatum* was isolated from 22 of 50 cats and from 22 of 50 dogs obtained from Loudoun County, Virginia. Histoplasma was isolated most frequently from the cervical and peribronchial lymph nodes of both cats and dogs. Dissemination of the organism, as indicated by multiple positive cultures from numerous tissues, was present in 8 cats and 9 dogs, though none of these animals was considered to be clinically ill with histoplasmosis.

Only one cat had a positive skin test. None of the cat serums had agglutinins for collodion particles sensitized with histoplasmin or complement-fixing antibodies for the whole yeast phase antigen of *H. capsulatum*. No characteristic gross or histopathological lesion was associated with isolation of *H. capsulatum* from the cats. Histoplasma was not found in histologic sections from any of the cats.

Fourteen dogs had positive skin tests, 27 dogs had agglutinin titers of 1:20 or

higher for collodion particles sensitized with histoplasmin, and 15 dogs had complement-fixation titers of 1:15 or higher for the whole yeast phase antigen of *H. capsulatum*. Neither the skin test nor either of the serologic tests gave a good index of whether or not *H. capsulatum* could be isolated from the dog. A variety of intrapulmonary and peribronchial lymph node lesions were found, but it was not established whether any of these lesions were caused by *H. capsulatum*. Histoplasma was found in histologic sections from 8 of the 50 dogs. The organism was demonstrated in sections from 7 of these 8 dogs only by the use of a stain for polysaccharide.

The limitations of the methods used for skin tests, serologic tests, and for obtaining cultures for fungi from animal tissues are discussed.

# THE EFFECT OF MALARIA (*PLASMODIUM BERGHEI*) ON LEUKEMIA $L_{1210}$ IN MICE

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A relationship between malaria and leukemia was suggested in 1872 by Mosler, who believed that attacks of malaria might be a predisposing cause of leukemia.[1] On the other hand, temporary clinical improvement of leukemia has been reported to follow the artificial introduction of malaria into patients with leukemia[2] and mycoses fungoides.[3] The evidence has been most recently summarized by Forkner.[4] Additional evidence of a relationship between malaria infection and tumor growth has been offered by Trager,[5] who reported the inhibitory effect of *Plasmodium lophurae* infection on the growth of chicken tumor I. More recently, several antimetabolic drugs have been shown to inhibit the growth of the malaria parasite as well as that of certain transplantable tumors.*[6–10] This suggested that some of the growth requirements of the leukemia cell and the malaria parasite might be similar and if so, competition for such growth factors might develop were both diseases introduced into the same laboratory animal. A study of the effect of malaria infection (*Plasmodium berghei*) on the survival time and tumor size in mice bearing the transplantable leukemia $L_{1210}$ became practicable because of the spread in survival time between malarial deaths (20 days)[11] and leukemia deaths (10

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days)[12] in BD $F_1$ hybrid mice (C57 Black ♀ × DBA ♂).

## METHOD

Three hundred and ten BD $F_1$ mice, 10 to 20 weeks old and weighing between 20 and 26 g, were used in 12 series of experiments. Six to ten animals were housed in plastic cages and given Purina rabbit chow, Derwood checkers and water ad libitum. Animals of similar age and weight were used in each of the series. Animals were selected at random for inoculation in all series. Aseptic techniques were employed.

*Transplantable lymphoid leukemia.*—The origin of leukemia $L_{1210}$ has been described.[12] Transplants were made subcutaneously into the axillary space by inoculating 0.1 cc of a 1:4 to 1:8 dilution of a brei of the tumor made up in Locke's solution.

*Malarial infection.*—The origin of *P. berghei* has been described.[13] The course of the infection in BD $F_1$ mice is considerably longer than in DBA, C57 Black[11] or in white mice.[14] Each mouse received 0.1 cc of a 1:1000 saline dilution of heart's blood obtained from a donor mouse infected with the Kasapa strain of *P. berghei* maintained by blood passage. Parasite counts were made from Giemsa-stained smears at irregular intervals in each series.

In three of the series the animals were infected with malarial parasites *before* receiving the transplantable tumor. In nine other series the animals were infected with malaria *after* receiving the tumor transplant. Survival was calculated with the date of transplantation of leukemia $L_{1210}$ taken as day 0. One survival day was credited to each animal for each day of survival, beginning at 9 a.m. of day 1.

At autopsy examination, sections from the transplantation site, bone marrow, spleen, liver, axillary lymph nodes, heart, lung, intestinal tract, genitourinary tract and brain were fixed in Zenker's formol solution or in 10% neutral formalin and stained with hematoxylin and eosin, and by the periodic acid Schiff and Giemsa methods.

In addition to the conventional methods of statistical analysis, the data were analyzed by a nonparametric significance test based on ranks, according to the method of Festinger,[15] as suggested by Mr. Marvin Schneiderman, Biometrics Section, NCI.

## RESULTS

All animals receiving the transplantable tumor developed leukemia during the experiment, confirmed by examination of histological sections or of peripheral blood smears. All animals infected with malaria parasites developed malaria, confirmed by study of peripheral blood smears. All animals succumbed to one or to both diseases. In three series of experiments (34 to 45 animals each) based on 116 mice in which malarial infection was introduced *prior* to the transplantation of leukemia, no significant difference was detected in the mean survival between mice with leukemia alone (10.5 days) and mice with both malaria and leukemia (10.7 days). In each of nine other series (14 to 33 animals each) including 208 animals, in which the malarial infection was introduced *after* leukemia had developed, mice with both diseases survived longer ($P < 0.01$). The mean duration of survival of these nine groups of leukemic control mice was 10.49 days (S.E. = 0.49) after transplantation, while that of mice with both leukemia and malaria was 12.52 (S.E. = 0.30) (table 1).

Graphs (fig. 1) depicting the duration of survival reveal peaks on the 9th and 10th days for those 2 groups with leukemia alone, with 45% of the animals surviving 10 days. In the group receiving malaria *before* leukemia there are peaks on the 10th and 12th days,

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TABLE 1.—*The effect of* P. berghei *on the duration of survival of mice bearing transplanted leukemia* $L_{1210}$.

| Dilution $L_{1210}$ (inoculation day 0) | Number of animals | Mean survival (days) | S.E. | *P. berghei* inoculation day | Number of animals | Mean survival (days) | S.E. |
|---|---|---|---|---|---|---|---|
| 1:6 | 14 | 10.5 | 0.28 | 0 | 20 | 10.4 | 0.30 |
| 1:5 | 11 | 12.1 | 0.36 | −1 | 26 | 12.0 | 0.16 |
| 1:5 | 14 | 9.0 | 0.28 | −5 | 31 | 9.6 | 0.09 |
| Total | 39 | | | | 77 | | |
| Mean | | 10.53 | | | | 10.67 | |
| 1:5 | 13 | 12.0 | 0.44 | +2 | 13 | 13.5 | 0.42 |
| 1:5 | 6 | 10.5 | 0.40 | +3 | 7 | 12.1 | 0.55 |
| 1:4 | 10 | 9.3 | 0.15 | +1 | 20 | 11.8 | 0.44 |
| 1:8 | 10 | 13.2 | 0.51 | +3 | 10 | 14.5 | 0.49 |
| 1:8 | 10 | 10.9 | 0.41 | +3 | 10 | 12.0 | 0.56 |
| 1:4 | 10 | 9.8 | 0.25 | +3 | 10 | 12.6 | 0.36 |
| 1:4 | 10 | 8.4 | 0.45 | +3 | 10 | 13.2 | 0.45 |
| 1:5 | 10 | 9.7 | 0.21 | +3 | 16 | 11.7 | 0.45 |
| 1:6 | 14 | 10.5 | 0.35 | +2 | 19 | 11.2 | 0.26 |
| Total | 93 | | | | 115 | | |
| Mean | | 10.49 | | | | 12.52 | |

with 50% of the animals surviving the 10th day. In the group receiving malaria *after* leukemia there is a peak on the 12th day, with 94% of the animals surviving the 10th day. Of the animals receiving only leukemia, 14% survived beyond the 10th day, in contrast to 4% of those receiving malaria *before* leukemia and to 35% of those receiving malaria *after* leukemia.

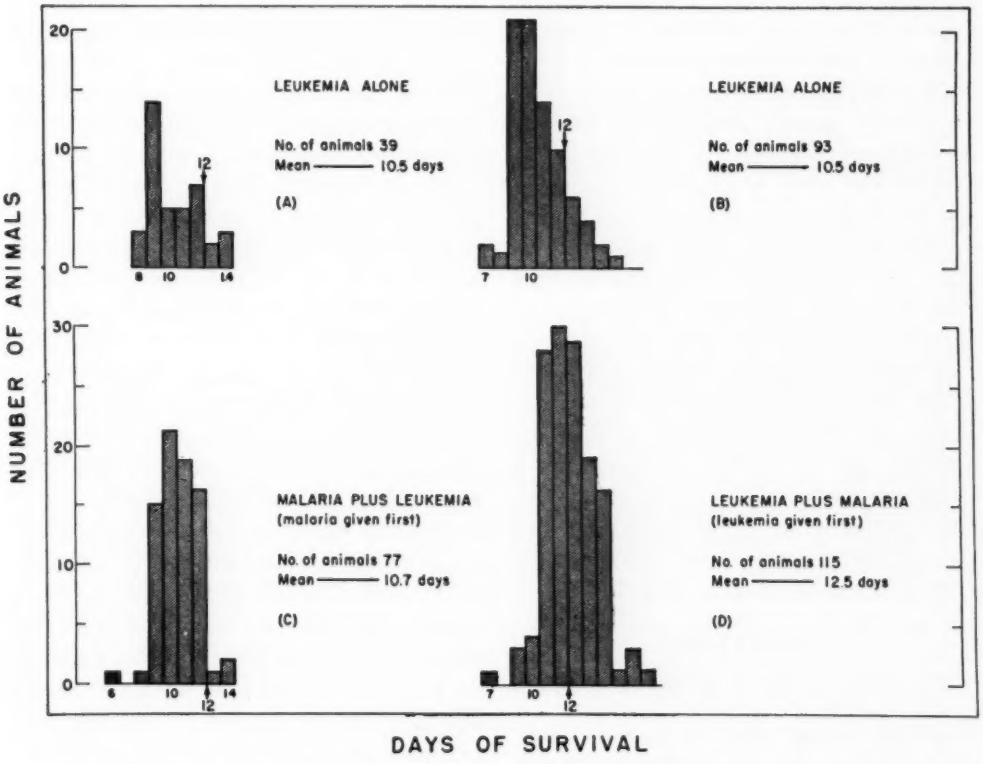


FIG. 1.—Survival of mice inoculated with leukemia (A and B) compared to that of mice incoulated with both leukemia and malaria (C and D).

TABLE 2.—*The effect of* P. berghei *on the weight of the spleen and of the tumor mass in mice bearing transplanted leukemia* $L_{1210}$.

| *Leukemia* | | | | *Leukemia plus malaria* | | | |
|---|---|---|---|---|---|---|---|
| Animal no. | Weight, g | Tumor weight, mg | Spleen weight, mg | Animal no. | Weight, g | Tumor weight, mg | Spleen weight, mg |
| 1 | 24 | 1117 | 372 | 11 | 23 | 686 | 236 |
| 2 | 27 | 1058 | 350 | 12 | 25 | 604 | 254 |
| 3 | 22 | 1033 | 264 | 13 | 23 | 704 | 248 |
| 4 | 23 | 954 | 264 | 14 | 24 | 628 | 371 |
| 5 | 24 | 733 | 384 | 15 | 24 | 606 | 374 |
| 6 | 24 | 1128 | 314 | 16 | 21 | 652 | 254 |
| 7 | 23 | 1107 | 382 | 17 | 21 | 590 | 272 |
| 8 | 23 | 1112 | 304 | 18 | 19 | 507 | 312 |
| 9 | 23 | 973 | 312 | 19 | 23 | 702 | 315 |
| 10 | 25 | 857 | 360 | 20 | 22 | 731 | 312 |
| Mean | 23.8 | 1007.2 | 330.6 | | 22.4 | 641.0 | 284.8 |
| S.E. | 0.5 | 39.4 | 2.0 | | 0.6 | 22.4 | 3.8 |

Series 53-6. $L_{1210}$ transplanted S.C. 3/5/53 with 0.1 cc of a 1:4 brei in Lockes' solution. *P. berghei* inoculated I.V. 3/6/53 with 100,000 parasitized erythrocytes. Positive malaria smear 3/11/53. Body weight at beginning of experiment 23 g in each of 20 mice.

Study of the histologic sections offered no clue for the cause of the increased survival. However, the BD $F_1$ animals with malaria and leukemia were observed to die with tumors considerably smaller than animals with leukemia alone. Tumor growth was then measured in three additional series of leukemic DBA mice sacrificed 6 to 8 days after transplantation and 1 to 3 days after inoculation with *P. berghei*. Animals were killed by cervical dislocation. The mass of lymphomatous tissue growing in the axillary region was excised, weighed on a Roller-Smith balance and compared with the tumors removed from similarly handled nonmalarial control mice with leukemia. In each of the three series the mean weights of the tumor masses and of the spleens were less in the animals with both malaria and leukemia, in contrast to those in mice with leukemia alone. However, only in one of the three series was this difference statistically significant at a level of $P$ less than 0.01.*

## DISCUSSION

The data in these 12 series provide evidence of a small inhibitory effect of a *P. berghei* infection on the speed of the lethal effect of leukemia $L_{1210}$. BD $F_1$ mice inoculated subcutaneously with a brei of transplantable leukemia $L_{1210}$ survived 10.5 days. When malaria infection was introduced intravenously before tumor transplantation, animals survived 10.7 days. When malaria infection was introduced after tumor transplantation in each of nine series of experiments, animals with both diseases lived slightly longer (12.5 days) than control animals with leukemia alone (10.5). This slight postponement of leukemic death is statistically significant. Its biologic significance is yet to be determined.

Either disease results fatally, and there is an overlap in the duration of survival in animals with only malaria or leukemia. From related studies it is known that BD $F_1$ mice will die from malaria as early as the 6th day. Thus some of the earlier deaths in animals with both diseases were probably due to malaria alone. In addition, from the related studies on the course of malaria in BD $F_1$ mice it can be anticipated that 18% of the deaths during the first 14 days were probably due to malaria only. Nevertheless, despite the added handicap of overlapping malarial

* In this last series (table two) the animals were sacrificed 8 days after transplantation of leukemia and had received an inoculum of 100,000 malaria parasites on the 1st day after transplantation.

deaths, animals with both malaria and leukemia survived as long or longer than animals with leukemia alone.

Weight loss and loss of appetite are often considered to beneficially affect the survival time in tumor-bearing animals. However, malaria does not adversely affect the appetite or weight of BD $F_1$ mice until late in the course of the disease. Were weight loss present and if of significance in survival in this experiment, one might expect a greater survival in animals receiving malaria before receiving leukemia, for in this case the duration of malaria infection would be longer. This was not observed. Others have reported that restricted food intake[16] and weight loss[12] do not increase the survival of mice bearing leukemia $L_{1210}$. In addition, although a marked anemia develops in the course of malaria, no decrease in survival was noted in the groups with malaria and leukemia. The absence of either a decrease or an increase in survival time in animals receiving malaria prior to leukemia can be offered as suggestive evidence against both the presence of a nonspecific toxic effect and a nonspecific sparing effect attributable to the malaria infection.

The data presented in this report offer tenuous evidence of the hypothesis originally offered, i.e., the malaria parasite and the leukemia both have certain similarities in their growth requirements. Additional data based upon the effect of malaria on the course of a spectrum of tumors are needed. Attention should be directed to the effect, if any, of malaria on the course of those tumors resembling leukemia $L_{1210}$ in response to folic acid analogues as well as to those tumors known to be unresponsive to such antagonists. In addition, knowledge of the effect of other parasitic infections, related to malaria biologically or in pharmacological responses, would also be pertinent in the evaluation of the hypothesis. It is interesting to note that very recently Trager and McGhee have reported the inhibition of chicken tumor I after intravenous injection of large amounts of parasite-free plasma prepared from birds infected with *P. lophurae*. Such plasma was most effective when collected just prior to the peak parasitemia.[17] This suggests that an active malarial infection may bring about the development of tumor-inhibiting factors in the plasma in a manner similar to the development of bactericidal substances in the serum during the acute phase of certain infectious diseases.[18]

16. Greenspan, E. M., Leiter, J. and Shear, M. J. 1950, Effect of alpha-peltatin, beta-peltatin and podophyllotoxin on lymphomas and other transplanted tumors. J. Nat. Cancer Inst. **11**: 1295–1332.

17. Trager, W., and McGhee, R. B. 1953, The inhibition of chicken Tumor I by plasma from chickens infected with malaria parasite *Plasmodium lophurae*. Fed. Proc. **12**: 405.

18. Good, R. A. 1952, Acute phase reactions in rheumatic fever. In symposium on rheumatic fever, Lewis Thomas, editor, University of Minn. Press, pp. 115–135.


## SUMMARY

Three hundred and ten BD $F_1$ hybrid mice (C57 Black ♀ × DBA ♂) were used in 12 series of experiments to study the effect of malaria infection (*Plasmodium berghei*) on the survival of mice bearing the transplantable leukemia $L_{1210}$. Leukemia control mice survived for a mean of 10.5 days. In 3 series of experiments in which malaria was introduced prior to leukemia, animals survived 10.7 days. In each of 9 series of experiments in which malaria was introduced after leukemia was established, animals with both diseases lived longer and survived a mean of 12.5 days.

# DIFFERENCES IN SURVIVAL OF SEVERAL INBRED STRAINS OF MICE AND THEIR HYBRIDS INFECTED WITH *PLASMODIUM BERGHEI*

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In an earlier paper[1] we found that C57 Black mice inoculated with *Plasmodium berghei* lived significantly longer than similarly inoculated DBA mice. Their hybrids, BD $F_1$, lived significantly longer than either parent. Since the parasitemia increased at about the same rate in both parents and their hybrid, we believed we were dealing not with resistance to the parasite itself but with resistance to causes of death from malaria. While there were differences in survival within the strains due to the sex and age of the mice, these were smaller than those attributable to the differences between strains.

The study has now been extended to include nine additional inbred strains of mice and eight additional hybrids.

## MATERIALS AND METHODS

The Kasapa strain of *P. berghei* was used throughout. The strain was maintained in this laboratory by weekly passages of infected blood through general purpose albino Swiss mice. The Swiss mice served as donors of infected blood for all the experimental mice. Heart blood was diluted with physiological saline so that each 0.1 ml contained 10,000,000 parasitized erythrocytes. Each experimental mouse received 0.1 ml of inoculum through a tail vein. Blood smears were prepared and examined from each mouse on the 3rd or 4th day after inoculation to establish the presence of an infection. All the mice were found infected when so examined.

Mice of both sexes, 3 to 12 months old, were used. Five or six groups of mice, each of a different strain or hybrid and usually infected by the same inoculum on the same day, formed a single series. In general, the groups comprising a series were formed on the basis of availability of the mice. By this method mice of each strain were tested in at least two, usually more, independent series. In each series, the mice of each strain were represented by 4 to 15 individuals, again depending on availability.

## RESULTS

The survival data for each strain have been pooled and the strains have been arranged from the shortest- to the longest-lived (table 1). In table 1 are given the number of series and the number of mice of each strain used, the mean survival in days, the standard error of the mean, the time in days for 50% mortality, and the range in survival. C57 Black mice survived almost twice as long as A or SWR mice; mice of the other strains had intermediate survival rates. There was a wide range of survival in each strain, as well as a considerable overlap of the ranges even of the shortest- and longest-lived strains. The time of 50% mortality forms a small increment progression among the strains, similar to that formed by the

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† Laboratory of Pathology, National Cancer Institute.

1. Greenberg, J., Nadel, E. M. and Coatney, G. R., 1953, The influence of strain, sex and age of mice on infection with *Plasmodium berghei*. J. Infect. Dis. **93**: 96–100.

TABLE 1.—*Survival of various strains of inbred mice infected with* P. berghei.

| Strain* | Series | Number of mice | Mean survival (days) | Survival range (days) | 50% mortality (days) |
|---|---|---|---|---|---|
| A/LN | 5 | 38 | 8.38±0.68 | 5–17 | 6 |
| SWR | 4 | 21 | 8.81±0.37 | 6–13 | 8 |
| $C_3H/H$ | 3 | 23 | 9.65±0.54 | 6–15 | 9 |
| STR/SN | 10 | 77 | 10.45±0.38 | 6–20 | 10 |
| BRUNT/STR | 4 | 31 | 10.71±0.42 | 8–17 | 10 |
| C58/LN | 2 | 17 | 10.40±0.45 | 6–15 | 11 |
| BALB/C | 7 | 65 | 12.51±0.28 | 8–17 | 12 |
| C57 Brown | 2 | 18 | 12.33±0.60 | 7–17 | 12 |
| RIL(AKR/LW) | 2 | 18 | 14.39±0.51 | 8–17 | 15 |
| C57 Leaden | 2 | 16 | 15.00±0.89 | 8–20 | 14 |
| C57 Black | 8 | 51 | 17.60±0.64 | 8–30 | 18 |

* Unless otherwise indicated, inbred mice were supplied from the mouse inbred nucleus of the Laboratory Aids Branch, National Institutes of Health.

mean survival. A statistical analysis of the data revealed the following: There was no significant difference in survival ($P>0.01$) between C57 Black and C57 Leaden; BALB/C and C57 Brown; Brunt, STR/SN, and C58; C3H and DBA (from previous data), and SWR and A. The results of each of these pairs or triplets were significantly different from those of all other strains of mice tested, except that the results with RIL mice were not significantly different from those of C57 Leaden or C57 Brown.

Data on the survival of hybrids are given in table 2. Again the mean survival of the longest-lived hybrids was twice that of the shortest-lived hybrids. It should be noted that C57 Black or C57 Leaden mice were one of the parents of some of the longer-lived hybrids as well as the parents of the shortest-lived hybrids. The mean survival of shortest-lived hybrid, C57 Black×C57 Leaden, was significantly less than that of either parent. The BALB/C×A/LN and C57 Black×C57 Brown hybrids did not live significantly longer than the longer-lived parent; all other hybrids lived significantly longer than the longer-lived parent.

## DISCUSSION

There has been a great deal of work on resistance of mice to infectious diseases (see Grüneberg[2]). However, the results presented in this and our previous paper[1] differ from most earlier work in one or more of the following respects: (1) we used a protozoan parasite; (2) the strains of mice we used were not specifically selected because of resistance to malaria; rather, they were inbred for characteristics with no obvious connections with malaria; (3) the differences observed were probably not due to resistance to the entry or growth of the parasite, but to causes of death related to the generalized disease process; finally, (4) hybrids were not intermediate between the parents in their survival against malarial infection; most were longer-lived than either parent, some behaved much as the longest-lived parent, and one hybrid was, on the aver-

2. Grüneberg, H. 1952, The Genetics of the Mouse, The Hague, Martinus Nijhoff.

TABLE 2.—*Survival of various hybrids of inbred strains of mice infected with* P. berghei.

| Hybrids | Series | Number of mice | Mean survival (days) | Survival range (days) | 50% mortality (days) |
|---|---|---|---|---|---|
| C57 Black×C57 Leaden | 3 | 25 | 11.28±1.04 | 5–23 | 10 |
| BALB/C×A/LN | 2 | 30 | 13.23±0.89 | 6–22 | 14 |
| C57 Black×C57 Brown | 5 | 58 | 15.05±0.55 | 7–24 | 15 |
| BALB/C×$C_3H$ | 3 | 30 | 15.10±0.42 | 11–20 | 16 |
| A/LN×DBA | 5 | 57 | 15.07±0.75 | 5–25 | 17 |
| BALB/C×DBA | 2 | 13 | 16.38±0.27 | 15–18 | 16 |
| C57 Leaden×A/LN | 4 | 31 | 19.29±0.76 | 9–26 | 20 |
| C57 Black×DBA | 7 | 44 | 21.23±0.45 | 13–29 | 21 |
| C57 Black×A/LN | 4 | 34 | 22.65±0.98 | 8–29 | 24 |

age, significantly shorter-lived than either parent.

Of some interest is the great variability of data within a strain. Most of the strains had a range of survival of 9 to 12 days, and one, C57 Black, had a range of 22 days, practically the entire range of the total population of mice examined. The earliest deaths among all the strains came within 3 days of each other, and there was little correlation within the entire range of strains between mean survival and longest survival, although those with the highest mean did show the highest survival time. The wide range in survival suggests the existence of uncontrolled extragenetic factors. Some of the variability may be attributable to the methodology, some may have been due to variability in the parasite, and some to such factors as age and sex. However, it should be mentioned that such data as we have show that there is little significant reduction in variability even if one confines his observations to a single strain of mice of one sex, or age, or litter, or inoculation series. The sources of variability within a single strain would require further investigation.

The ten inbred lines tested can be grouped into five or six phenotypic classes according to mean survival time. This, together with the fact that many hybrids lived longer than either parent, suggests that if genetic factors influence survival with malaria, there are several factors involved. That there may be more different genetic combinations than are indicated by the number of phenotypic classes is suggested by the results with C57 Black and C57 Leaden. These strains appeared to have similar survival rates, yet the significantly lower rate of their hybrids points to a possible genetic difference between the parents in this regard.

## SUMMARY

Ten inbred strains of mice and nine hybrids from these strains were infected with *Plasmodium berghei*, and their survival observed. The shortest-lived strain (A/LN) survived a mean of 8.38 days, the longest-lived (C57 Black), 17.6 days. Other strains were intermediate in their survival against malaria. Most hybrids survived, on the average, longer than either parent; two hybrids (BALB /C×A/LN and C57 Black×C57 Brown) did not live longer than the longer-lived parent, and one hybrid (C57 Black ×C57 Leaden) was significantly shorter-lived than either parent.

CORRECTION

Volume 94, Number 2

March–April, 1954

p. 141, line 25.—Change *identified* to *differentiated.*
p. 142.—The last sentence should read "The pH of the unadjusted medium after sterilization is 6.9."
p. 143, line 5.—Change *1.2 ounce* to *[illegible] ounce.*

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